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Effects of the TYR-MIF-1 Peptides on the Expression of CB1 Cannabinoid Receptors in the Rat Mesencephalic Trigeminal Nucleus Following Thermal Stress

Angel Dandov[1], Dimitrinka Atanasova[2,3], Nikolai Lazarov[1,3]*

[1] Department of Anatomy and Histology, Medical University of Sofia, Sofia, Bulgaria
[2] Department of Anatomy, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria
[3] Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

* Corresponding author e-mail: didiatan@bio.bas.bg; didiatanasova7@gmail.com

The present study aims at elucidating the role of TYR-MIF-1 peptides in the expression patterns of cannabinoid type 1 (CB1) receptors in the mesencephalic nucleus of the trigeminal nerve (Me5) in rats after mild thermal stress. Immunohistochemistry revealed that in rats subjected to short-term heat stress the application of the CB1 agonist arachidonoylethanolamide (AEA) induces the intense expression of CB1 receptors in the Me5. Moreover, treatment with the peptides of the Tyr-MIF-1 family results in a distinct alteration in the CB1 expression. Specifically, the administration of MIF-1 and Tyr-K-MIF-1 increases CB1 expression levels, while Tyr-MIF-1 and Tyr-W-MIF-1 decrease them. It is likely that the first two members directly influence the CB1 expression in the rat Me5, while the other two probably interact via second messengers or another neutotransmitter. The exact mechanism of the interaction is yet to be clarified.

Key words: CB1 cannabinoid receptor, mesencephalic trigeminal nucleus, Tyr-MIF-1 peptides, endocannabinoid system, thermal stress

Introduction

The mesencephalic nucleus of the trigeminal nerve (Me5) is a unique brain structure with an unusual central location and a noticeable ability for adequate adaptive morphological and neurochemical responses to changes in the environment [1]. The Me5 is made up of

two distinct subpopulations of neurons, most of which are large-sized pseudounipolar while a few are small in size and of spherical or ovoid shape [1]. These form a band extending from the lateral border of the periaqueductal gray (PAG) to the trigeminal motor nucleus. The structure also receives input from the mechanoreceptors of the periodontal ligaments and innervates the jaw-closing muscles [3, 14]. Me5 neurons are located in the brainstem and are influenced by various brain regions regulating feeding behavior [4].

The endocannabinoid system (ECS) is proposed to be involved in the regulation of a variety of physiological processes. It is composed of lipid-derived neurotransmitters, called endocannabinoids, that bind to two G-protein-coupled receptors, the cannabinoid type 1 (CB1) and type 2 (CB2), and cannabinoid receptor proteins [2, 7, 9, 12]. CB1 is a pre-synaptic G protein-coupled heteroreceptor that is expressed in the central and peripheral nervous system of rats [13, 15]. Moreover, it is highly expressed in rat brain areas that are involved in pain modulation [11, 12, 16] and is activated by endocannabinoids that include N-arachidonoylethanolamide (anandamide, AEA). Our previous study has revealed that the application of AEA evokes a strong expression of CB1 in the Me5 in rats exposed to short-term thermal stress [6].

The Tyr-MIF-1 family consists of four peptides, i.e. MIF-1 (Pro-Leu-Gly-NH_2), Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2), Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2), and Tyr-K-MIF-1 (Tyr-Pro-Lys -Gly-NH_2). MIF-1 does not bind to opioid receptors, while Tyr-K-MIF-1 binds only to its own non-opioid receptor. The other two peptides, Tyr-MIF-1 and Tyr-W-MIF-1, possess both opioid and non-opioid binding sites [10].

Since the ECS modulates stress reaction, it would be intriguing to find out the possible interactions between the above-mentioned peptides and the ECS after heat stress exposure. Therefore, we set it as a goal of this study to investigate the effects of the Tyr-MIF-1 peptides on the expression of CB1 cannabinoid receptors in the rat Me5 following thermal stress.

Materials and Methods

Animals

The experiments were carried out on 25 male Wistar rats, weighing 180-200 g, kept at an optimal room temperature of 22°C and under normal conditions. The animals were divided into 4 experimental and 1 control groups, each including 5 animals (n=5). The study was approved by the Institutional Ethics Committee at the Institute of Neurobiology of the Bulgarian Academy of Sciences. All experimental procedures were performed in agreement with the European Communities Council Directive 2010/63/EU for the protection of animals used for scientific purposes.

Acute experimental model of heat stress

The animals were kept for 1 hour at a high ambient temperature of 38°C. The rats moved freely in the thermal chamber but were not supplied with food or water during the heat exposure.

Administered drugs and treatments

CB1 receptor agonist AEA at a dose 1mg/kg b.w, dissolved in dimethyl sulfoxide (DMSO), was injected intraperitoneally. The peptides of the Tyr-MIF-1 family at a

dose 1mg/kg b.w. were dissolved in sterile saline solution (0.9% NaCl) and introduced intraperitoneally. The administered drugs used in this study were purchased from Sigma (Sigma Chem. Co., St. Louis, MO, USA).

Experimental design

The animals were divided into 5 groups and were subjected to heat stress for one hour. AEA was injected immediately after the stress. In the four experimental groups (1h HS+AEA+MIF-1; 1h HS+AEA+Tyr-MIF-1; 1h HS+AEA+Tyr-W-MIF-1; 1h HS+AEA+Tyr-K-MIF-1) the peptides of the Tyr-MIF-1-family were administered 10 min after the AEA application. On the other hand, the control group underwent only 1h HS+AEA treatment.

Immunohistochemical procedure

The rats were deeply anesthetized with ketamine-xylazine at the appropriate dosage for rats and perfused first with 0.05 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde (PFA) in 0.01 M phosphate buffer (PB), pH 7.4. The brain was dissected out and postfixed in the same fixative overnight at 4ºC. Thereafter, the tissues were embedded in paraffin and cut into 5 µm thick sections. The samples were then deparaffinized and subsequently processed for immunohistochemistry using an ImmunoCruz™ goat ABC Staining System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). In brief, the sections were treated with 1% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase. The background staining was blocked with 5% normal goat serum in PBS for 1 hour. Subsequently, they were incubated with a polyclonal goat anti-CB1 receptor antibody (1:500, Santa Cruz Biotechnology) overnight at 4°C in a humid chamber, followed by donkey anti-goat IgG (1:500, Santa Cruz Biotechnology) for 2 h at room temperature, and lastly the AB enzyme reagent was applied for 30 min at room temperature. The peroxidase activity was visualized by diaminobenzidine as a chromogen. Finally, the sections were dehydrated, cleared in xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

The specificity of the immunostaining was controlled by the replacement of the primary antibody with PBS.

Photodocumentation and image processing

The immunostained sections for the CB1 receptor were digitalized using a Nikon DXM1200c research microscope (Nikon Inc., Tokyo, Japan) equipped with a DMX 1200 digital camera. The digitized images were captured with an objective lens 40x and a total magnification 400x. Prior to use, the system underwent an accurate calibration to correct the captured images. For every single image, the camera settings and light source were kept the same. The digital images were saved in TIF format.

Densitometric analysis and statistics

The CB1 immunostaining intensity was assessed on binary converted images using the semi-automated densitometric evaluation after a threshold was set via the program ImageJ 1.48v (NIH, Bethesda, MD, USA). Staining intensities were semi-quantified and were presented as percentage areas. Two blinded researchers performed the evaluations and the obtained results were averaged.

The statistical analyses were performed using the GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The data were presented as means±standard error of the mean (SEM). An unpaired *t*-test for Gaussian distributed data and Mann-Whitney *U*-test for non-Gaussian distributed data were accomplished. The results were considered statistically significant when $p<0.05$.

Results

The immunohistochemical experiments revealed immunostaining intensity for CB1 in two distinct subpopulations of pseudounipolar neurons in the Me5. The immunopositive reaction was localized mainly in the cytoplasm of the cells while their nuclei remained negative. The perikarya and proximal processes of the neurons were differently immunostained in the examined experimental groups (**Fig. 1**). Specifically, following a 1-hour heat exposure with AEA (1h HS+AEA), intensely stained large and small ovoid pseudounipolar neurons were observed in the Me5 (**Fig. 1A, B**).

The results of the statistical analysis showed an increased CB1 expression in 1h HS+AEA+MIF-1-animals (**Fig. 1C; Fig. 2**) compared to 1h HS+AEA controls (**Fig. 1A, B; Fig. 2**) (69.7826±2.14 vs 53.4636±3.67; p=0.0001). However, we found a decreased CB1 expression in the groups that were also treated with Tyr-MIF-1 (**Fig. 1D; Fig. 2**) and Tyr-W-MIF-1 (**Fig. 1E; Fig. 2**), (34.3982±4.77 vs 53.4636±3.67; p=0.0057) and (19.2488±4.97 vs 53.4636±3.67; p=0.0003), respectively when compared to the control group of 1h HS+AEA-animals (**Fig. 1A, B; Fig. 2**). The administration of Tyr-K-MIF-1 (**Fig. 1F; Fig. 2**) significantly altered the expression

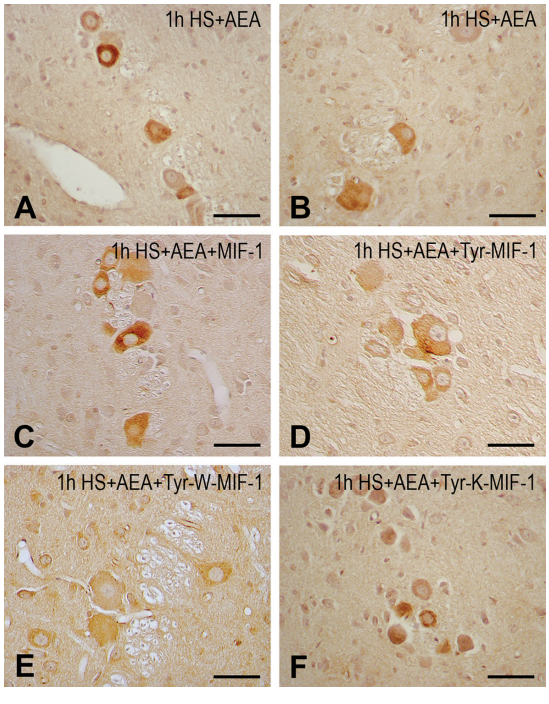

Fig. 1. Immunohistochemical expression of CB1 receptors in some large and small ovoid pseudounipolar neurons located in the mesencephalic trigeminal nucleus of rats. The cell bodies of these neurons are differently immunostained in the examined 5 groups. **(A, B)** Light photomicrographs showing CB1 receptor immunoreactivity in control group in which the animals are subjected to 1-hour of heat stress followed by CB1-receptor agonist anandamide (AEA). In the remaining groups four different peptides from the Tyr-MIF-1 family are administered, i.e. MIF-1 **(C)**, Tyr-MIF-1 **(D)**, Tyr-W-MIF-1 **(E)** and Tyr-K-MIF-1 **(F)**, respectively. Note that the administration of both MIF-1 **(C)** and Tyr-K-MIF-1 **(F)** enhances the expression of CB1 receptors in the examined area while the peptides Tyr-MIF-1 **(D)** and Tyr-W-MIF-1 **(E)** cause decreased CB-1 receptor expression. Scale bars = 50 μm.

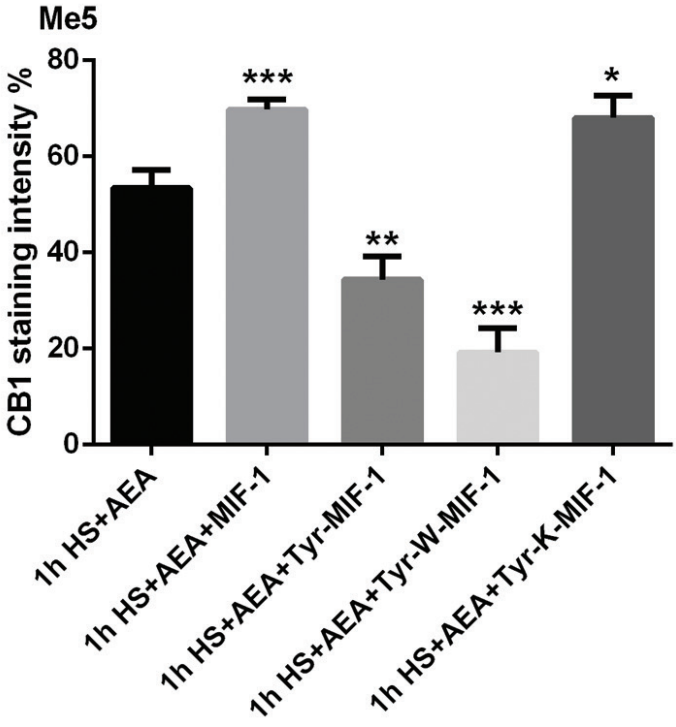

Fig. 2. Densitometric analysis of CB1 staining intensity in 1 control and 4 examined experimental groups in the rat mesencephalic trigeminal nucleus (Me5). The prominent statistically significant increase in the CB1 immunostaining was observed in groups 1h HS+AEA+MIF1 and 1h HS+AEA+Tyr-K-MIF1 compared to the control group. The data are presented as Mean+S.E.M. The level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of the CB1 receptor when compared to the non-peptide treated control group 1h HS+AEA (68.0626±4.63 vs 53.4636±3.67; p=0.0299).

Discussion

The results of this study show that the peptides of the Tyr-MIF-1 family interact differently with the ECS after an acute heat stress. The administration of each member of the family results in a distinct alteration in the expression of the CB1 receptor in the Me5. In particular, the administration of MIF-1 and Tyr-K-MIF-1 increases the CB1 expression under these conditions, while Tyr-MIF-1 and Tyr-W-MIF-1 decrease it.

Previous reports mainly focus on the distribution of the CB1 receptor in rat brainstem regions such as the PAG which is traditionally associated with pain transmission related to opioids [5, 16]. Data also show that under normal conditions this type of cannabinoid receptor is expressed only in the gray matter of the fourth ventricle in rats [13, 15]. Here we provide further evidence that the thermal stress induces such an expression in the rat

Me5 and that the administration of its agonist AEA at the end of the stress leads to some decrease in heat-induced analgesia.

The most important concept of the "gate control" theory of pain is that the pain sensation is subject to modulation, both by inputs from the periphery and by signals that come from the brain itself [8]. The rostroventromedial medulla and the PAG are thought to be areas that modulate pain and receive significant projections from the brainstem trigeminal nuclear complex [11]. This modulation is exerted by a presynaptic inhibition of primary afferent nociceptors and by post-synaptic inhibition of second-order trigeminal neurons via inhibitory interneurons [11].

In conclusion, it can be inferred that Tyr-MIF-1 and Tyr-W-MIF-1 have a direct impact on CB1 expression in the Me5, while MIF-1 and Tyr-K-MIF-1 probably interact via second messengers or through the activation of additional neurotransmitter system(s). Further research would clarify the nature of this input mechanism and could favor the development of new therapeutic strategies of pain management.

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