ALTERATION OF CB1 RECEPTORS DENSITY IN AMYGDALA AFTER STRESS AND KYOTORPHIN INJECTION

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ABSTRACT

Introduction: Induction of acute physical stress by low temperature or cold stress (CS) is one of the most commonly employed animal models for studying different aspects related to stress. Considerable work has established the amygdala as a key site involved in the generation of fear, anxiety and emotional responses to external stimuli. Also it is known that amygdala coordinates affective, autonomic, and behavioral responses. Literature and our data showed that the neuropeptide kyotorphin (KTP) may play a neuromodulatory role with regard to pain perception, stress respond, thermoregulation, and exploratory behavior. It was supposed that the mechanisms of its action are due to Met-enkephalin release and involvement of monoaminoergic neurotransmitter systems. Because the endocannabinoid system is strongly associated with the regulation of functions as learning and memory, pain perception and sensory physiology, the aim of our study was to examine and compare the effect of CS and KTP on density of CB1 receptors in the amygdala.

Materials and methods: Immunocytochemistry and morphometric analysis were used to determine density of CB_1-immunopositive neurons in the amygdala of male Wistar rats exposed to acute CS (1 hour, on 4^oC) and after intraperitoneal KTP injection.

Results: Data analysis revealed that both, CS and KTP applied alone significantly increased the density of CB1-immunopositive neurons in the rat amygdala. The quantity of immunoreactive cells in the rats, undergoing CS, demonstrated around 40% higher density of CB1-receptors compared with control group. The same effect was observed in group of rats injected with the neuropeptide, while the combination of both factors - CS immediately followed by KTP injection resulted in more pronounced (about 50%) increasing number of CB1-immunopositive neurons.

Conclusion: Our findings revealed that alternation in CB1 receptor density occurs in response to cold exposure as well as KTP injection, but the effect of both combined factors was stronger. We could suggest that activation of endocannabinoid system is one of the mechanisms by which stress and neuropeptides affect the synaptic connectivity between amygdaloid nuclei and lead to modulation of emotional behavior, learning, and stress-response.

Keywords*: cold stress, kyotorphin, endocannabinoids, amygdala*

Introduction

Living organisms are constantly challenged by external or internal challenges or stressors, which threaten homeostasis, defined as constancy of the internal environment [1, 13]. According to literature and our data, each type of stressor (immobilization, high or low temperature) has its own central neurochemical and peripheral neuroendocrine "signature", with quantitatively and qualitatively distinct mechanisms [2, 6]. Exposure to stress caused an array of biochemical, physiological and behavioral changes [14]. It alters the levels of many biologically active substances – hormones, mediators and neuropeptides.

One of the neuromodulators in the central nervous system is the neuropeptide termed "kyotorphin" – an endorphin-like substance, discovered in Kyoto. Literature data and our previous investigations showed that kyotorphin (KTP) bind to a specific receptor and play a role in pain regulation, thermoregulation, exploratory behavior and stress-induced analgesia [12, 19, 18, 21]. The physiological effects of Kyo fall clearly into two groups: those mediated via opioid peptides and opioid peptide-independent ones. According to several studies, the action of neuropeptide on

integrative brain functions in animals is due to its interaction with brain monoaminoergic neurotransmitter systems [8, 12].

Considerable work has established the amygdala as a key site involved in the generation of fear and anxiety responses, the assignment of emotional salience to external stimuli, the coordination of affective, autonomic, and behavioral responses [17].

It is also known that the endocannabinoid system (ECS) participates in multiple brain circuits implicated in stress reactions, learning, and extinction of fear, emotional regulation, and reward processes [7, 10]. The endocannabinoid CB1 receptors are found in many areas involved in regulation of the stress response including the paraventricular nucleus of the hypothalamus, the pituitary, adrenal glands but they are particularly concentrated in limbic structures such as the hippocampus and amygdala [20]. The CB_1 receptors main role appears to be the inhibition of various excitatory and inhibitory neurotransmitters. Their ligand-specific activation can modulate cognitive, memory and motor functions as well as analgesia [3, 16].

To our knowledge, no attention has been directed to the interaction between cold stress, KTP and cannabinoid systems in rats' amygdala. Therefore, the aim of our study was twofold: 1) to investigate the effect of KTP and CS applied alone on density of CB_1 immunopositive neurons in the rat amygdala; 2) to investigate the effect of both factors applied together.

Materials and methods

Animals and housing: The experiments were carried out on male Wistar rats (180-200g) kept under normal conditions at ambient room temperature (22°C). Each group included 6 rats. All experiments were performed during the light period between 09:00 and 13:00 h.

The experimental procedures were carried out in accordance with the institutional guidance and general recommendations on the use of animals for scientific purposes.

Acute model of cold stress: The animals were placed in a refrigerating chamber at 4^oC for 1 hour. The control group was not submitted to 1 hour stress procedure.

drugs and treatment: Kyotorphin (Bachem) was dissolved in sterile saline (0.9% NaCl) solution and was injected intraperitoneally (i.p) at a dose of 5 mg/kg. 10 min after neuropeptide injection the animals were anaesthetized with Thiopental (40 mg/kg, i.p.) and perfused through the heart with fixative (4% paraformaldehyde in 0.1M phosphate buffer, pH 7,2). Postfixation of the obtained brains was conducted in 4% buffered solution (0.1 M phosphate buffer, pH 7.4) of paraformaldehyde overnight at 4ºC. The brains were cryoprotected with 15 and 30 % saccharose. Small series of 40 μm thick coronal sections were cut on a freezing microtome from bregma -1.80 to bregma -4.16 according to stereotaxic athlas of the rat brain [15].

Immunohistochemistry: The free floating sections were collected in Tris-HCl buffer 0.05M, pH 7.6 and preincubated for 1 h in 5% normal goat serum in PBS. Afterwards, incubation of the sections was performed in a solution of the primary antibody for 48 hs at room temperature. We used a polyclonal rabbit anti-CB_1 antibody (Santa Cruz, USA), in a dilution of 1:1000. Then sections were incubated with biotinylated anti-rabbit IgG (dilution, 1:500) for 2 hs and in a solution of avidin-biotin-peroxidase complex (Vectastain Elite ABC reagent; Vector Labs., Burlingame CA, USA; dilution 1:250) for 1 h. This step was followed by washing in PBS and then in 0.05 M Tris-HCl buffer, pH 7.6, which preceded incubation of sections in a solution of 0.05% 3,3′-diaminobenzidine (DAB, Sigma) containing 0.01% H_2O_2 for 10 min at room temperature for the visualization. Afterwards, they were rinsed with 0.1 M Tris–HCl, pH 7.6 and thrice with 0.01 M PBS for 5 min and finally the sections were mounted on gelatin-coated glass, dried for 24 h and coverslipped with Entellan.

Morphometric and statistical analysis: Ten coronal sections from each brain were utilized for calculation of the neuronal packing density in amygdala of rats. The intensity of the staining was evaluated visually and number of CB_1 immunoreacrive neurons was counted. Paxinos and Watson's atlas (1986) [15] was used for anterior–posterior sites localization and the analysis was performed

using a microanalysis system. Data of the entire drawings were entered in computer programme (Olympus CUE-2), recorded automatically, calculated and statistically assessed by one-way analysis of variance (ANOVA) and post hoc Dunnett analysis were performed. All values are presented as mean ± standard error of the mean (SEM). Differences between the experimental groups were considered to be significant if $P \leq 0.05$.

Results

Immunohistochemistry was used to determine the cellular expression of CB_1-immunopositive neurons in the amygdala of male Wistar rats (Fig. 1A and 1B). Two experiments were included in this study. Experiment 1 examined the effect of neuropeptide KTP and the effect of CS applied alone on density of CB_1 immunopositive neurons in the rat amygdala. Experiment 2 was identical except that we studied the effect of two combined factors – CS and KTP injected immediately after stress procedure.

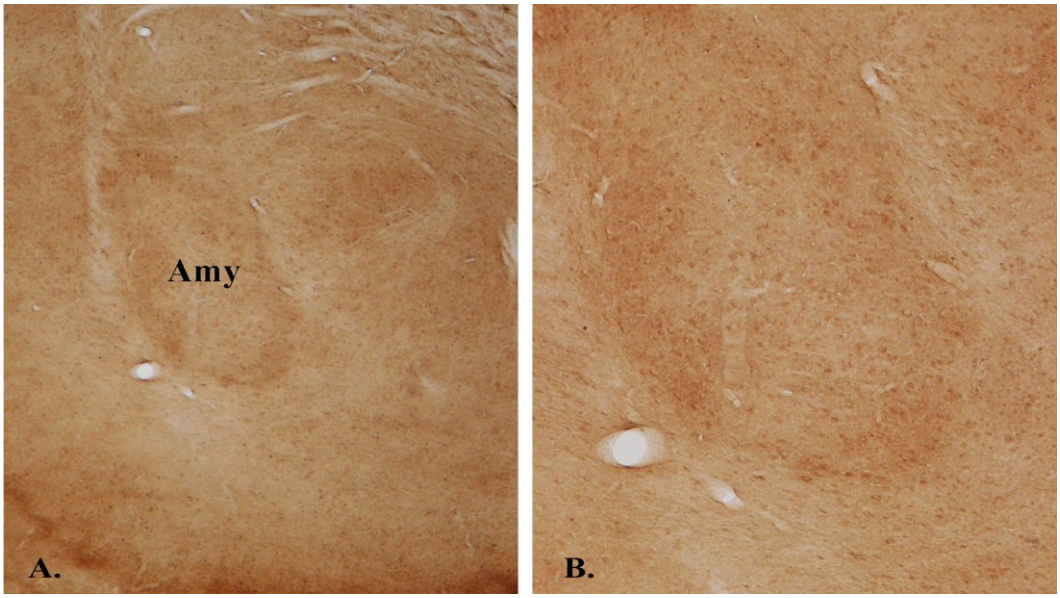

Fig. 1. Distribution of CB1 immunopositive neurons of the rat's amygdala. A. The borders of Amygdaloid body are well visible. Amy-Amygdala (x 40). B. Higher magnification. The immunoproduct is well visible around the cells (x 100).

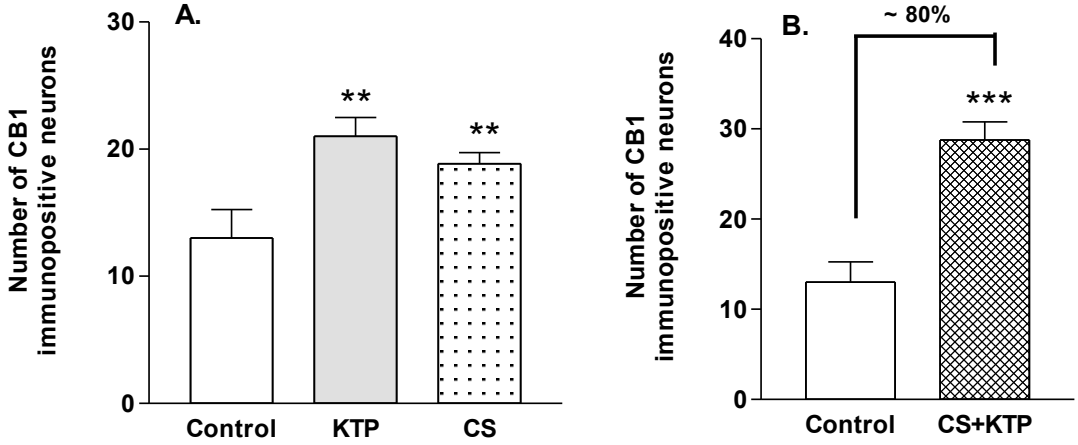

Fig.2. Effects of **A**. kyotorphin (KTP) and cold stress (CS) applied alone **B.** kyotorphin (KTP) applied immediately after cold stress (CS + KTP) on CB1 immunopositive neurons in male Wistar rat's amygdala. Mean values ± S.E.M. are presented **P < 0.01; ***P< 0.001 vs. control.

Our morphometric studies reveal differences between expressions of CB_1 immunopositive neurons in the experimental groups. In the first experiment CS applied alone significantly increased (approximately 40%) the density of CB_1 immunopositive neurons in the rat amygdala ($p < 0.01$) compared to the control. The same effect was observed when KTP was applied alone (Fig. 2A).

In the second experiment, the neuropeptide was injected immediately after stress and 10 min later rats were decapitated. In this case (CS+KTP) the vigorous expression of CB_1 immunopositive neurons (~ 80%) was observed compared to the control (Fig. 2B).

Discussion

Our results have shown that the amount of positively stained neuronal cell soma and processes found throughout the amygdala was significantly higher (~40%) in the treated with KTP rats and in rats following cold stress exposure compared to the controls. These data indicate that both factors, the KTP and CS are involved in the endocannabinoid signaling in the amygdala. Our results are with agreement with studies that the amygdala plays a pivotal role in highly adaptive phenomenon of stress. Lesions of this region were shown to result in striking behavioral changes, including visual agnosia, dietary changes, loss of conditioned fear and impairment of new fear learning. This observation set the stage for a line of research into the role of the amygdala in emotional memory that now spans multiple decades [9].

According literature data, the widespread distribution of CB_1 signaling underscores the pleiotropic functions of endocannabinoids in the regulation of motor control, learning and memory, pain, motivation, metabolic integration, and affects regulation [5]. CB_1 receptors are located mainly presynaptically and coupled to Gi/o-protein, inhibit via transient retrograde actions the release of different neurotransmitters, including GABA, glutamate, dopamine and serotonin [4, 11].

We suggest that positive effect of KTP on expression of CB_1 receptors of amygdala after stress are due to interactions of monoaminoergic and opioidergic neurotransmitter systems with endocannabinoid neurons.

To our knowledge, this is the first report showing the interaction between KTP and cannabinoid system. The involvement of neuropeptide in the endocannabinoid signaling and its strong effect on increased expression of CB_1 receptors is further evidence for neuropeptide essential role in regulatory effects of animal behavior during stress response.

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