



INCREASE IN THE NUMBER OF CB1 IMMUNOPosITIVE NEURONS IN THE AMYGDALOID BODY AFTER ACUTE COLD STRESS EXPOSURE

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ABSTRACT

PURPOSE: According literature data the animal's response to stress depends not only upon the state and conditions of the animal but also upon the nature of the stressor itself. It is known that stress have wide-ranging effects on neuroendocrine, autonomic, immune, and hormonal function. Different research groups have shown induction of acute physical stress by low temperature exposure which have been reported to impair motor activity, modulate pain perception, anxiety and depression-like behaviors in the animals. Considerable work has established the amygdaloid body 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. The aim of our study was to examine the effect of cold stress on density of CB1 receptors in the amygdaloid body.

METHODS: Immunocytochemistry and morphometric analysis were used to determined density of CB1 immunopositive neurons in the amygdaloid body of male Wistar rats exposed to acute cold stress.

RESULTS: Our morphometric studies reveal that cold stress significantly increased (around 40 %) density of CB1 immunopositive neurons in the rat amygdaloid body compared with control group.

CONCLUSIONS: Our results confirm that temperature fluctuation induces stress and endocannabinoid system is involved.

Key words: cold stress, endocannabinoids, amygdaloid body

INTRODUCTION

The term 'stress' generally indicates an internal (i.e., infection, psychological condition) or external (i.e., physical danger or damage) circumstance that threatens the homeostasis of the organism. Thus, stress results in a discrepancy, either real or perceived, between the demands of a situation and the organism's resources (1). Exposure to a stressor is immediately followed by somatic and neuro-physiological reactions involving peripheral organs such as adrenals, cardiovascular and respiratory systems, metabolism, and also brain

areas such as the prefrontal cortex, amygdala, hippocampus, nucleus accumbens, and hypothalamus (2). Considerable work has established the amygdaloid body 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.

It is known that the endocannabinoid system (ECS), formed by cannabinoid receptors (CB1 and CB2), their endogenous lipid ligands (endocannabinoids, ECBs), and the machinery for synthesis and degradation of ECBs plays a key role in the psychoneuroendocrine responses to stress. The ECS participates in multiple brain circuits implicated in stress reactions, learning, and extinction of fear, emotional regulation, and reward processes. The endocannabinoid CB1

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receptors are found in many areas involved in regulation of the stress response including the paraventricular nucleus of the hypothalamus, the pituitary, adrenal glands, and limbic-related areas of the brain (2-4).

We have previously found that following acute cold stress (CS) male Wistar rats showed a pattern of decreased anxiety-like behavior (reduced spontaneous and increased freezing behavior in the open field test) (5). Because the functional roles of amygdala and CB1 receptors in mediation of stress-induced anxiety are unclear, the aim of the current study was to examine the effect of cold stress on density of CB1 receptors in the amygdaloid body.

MATERIALS AND METHODS

Animals and housing: Twelve experimentally naive male Wistar rats (180-200g) served as subjects. They were housed in a temperature-controlled room ($22 \pm 2^\circ\text{C}$). Food and water were available *ad libitum*. The rats were randomly divided in two experimental groups: a cold stress (CS) group and a control group. 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°C . The control group was not submitted to 1 hour stress procedure.

Immunohistochemistry and morphometric analysis: The animals were anesthetized with thiopental (40 mg/kg b.w.). Transcardial perfusion was done with 4% paraformaldehyde in 0.1 M 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 4°C . 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 atlas of the rat brain (6). For 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-CB1 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 analysis was performed using a microanalysis system. Data of the entire drawings were entered in computer programme (Olympus CUE-2), recorded automatically, calculated and compared by Student's t-test.

RESULTS

A density of CB1 immunoreactive neurons was examined on high magnification. We found CB1 immunoreaction in cell bodies, axons and dendrites which was visualized as a brown color in perikarya and fibers. The cells bodies were comprised of several distinct shapes. Fine-beaded fibers and many puncta were observed around the neurons. CB1 receptor immunoreactivity in the amygdala in both groups of rats was diffuse and extensive. The most distribution of immunoreactive product was found around of the cell bodies. Great deal of CB1 receptor immunoreactivity was found outside of cell bodies labeling the processes of the cells.

Our morphometric studies reveal differences between expression of CB1 immunopositive neurons in control and experimental group (**Figure 1A, B**). Cold stress significantly increased the density of CB1 immunopositive neurons in the rat amygdala ($p < 0.05$) compared with controls. The quantity of immunoreactive cells in the rats, undergoing CS demonstrated approximately 40% higher density of CB1 receptors than control group (**Figure 1C**).

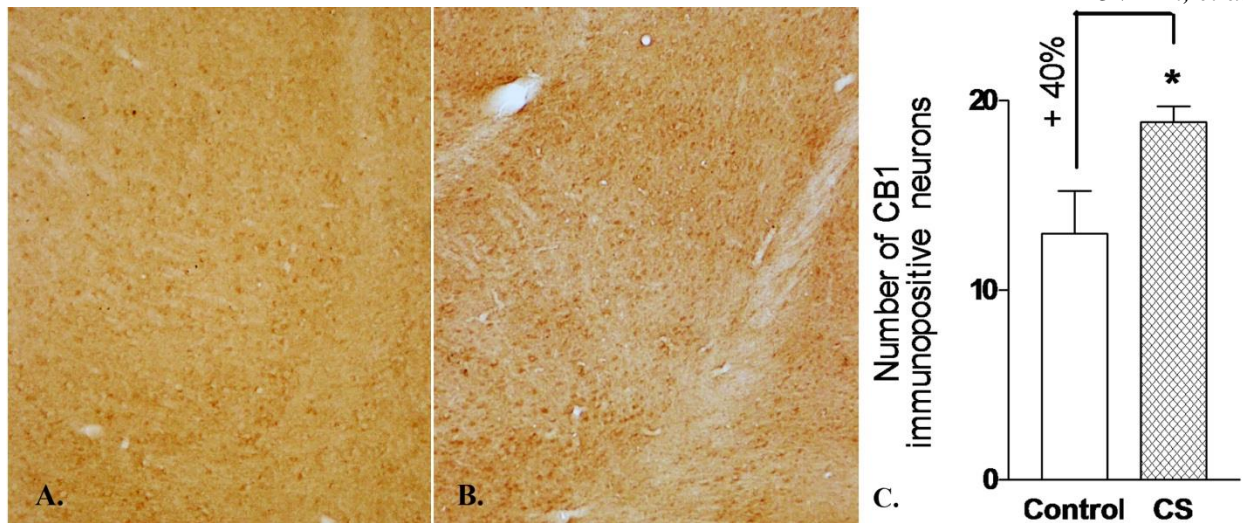


Figure 1. Photomicrograph of CB1 immunopositive neurons in the rat's amygdaloid body **A.** control group, **B.** cold stress group (x100); **C.** Effect of cold stress (CS) on CB1 immunopositive neurons in male Wistar rat's amygdaloid body. Mean values \pm S.E.M. are presented * $P < 0.05$ vs. control

DISCUSSION

Activation of the hypothalamic–pituitary–adrenal axis (HPA) by stress is thought to promote physiological and behavioral changes so that an organism can deal with a changing and challenging environment. It has been established that when animals are subjected to acute stress, a wide range of physiological alterations take place, and a short single intense exposure to stress can produce profound changes in an animal's stress response and behavior (7). A number of studies have revealed that various stressors produce differential effects, which are frequently referred to as stressor specific response (1, 2, 8). Cold stress is one of the most commonly employed animal models for studying different aspects related to stress (9). Acute change in temperature leads to stressful conditions by activation of temperature regulatory centre in the hypothalamus and subsequently HPA axis. Different research groups as well as our data have shown induction of acute physical stress by low temperature exposure which have been reported to impair motor activity, modulate pain perception, anxiety and depression-like behaviors in the animals (6, 10, 11). One of the main physiological mechanisms that organize the response to a stressful situation involves the amygdala - the main gate through which processed sensory information enters the emotional brain (3). The

amygdala is a limbic structure that receives monoaminergic innervation (dopamine, noradrenaline, serotonin, acetylcholine) as well as nerve afferents releasing glutamate from different areas of the brain (12, 13). Some literature data showed that in limbic-related areas of the brain CB1 receptors are found and ECs participates in multiple brain circuits implicated in stress reactions, learning, and extinction of fear, emotional regulation, and reward processes (3, 4). There is also evidence that endocannabinoid content of the amygdala is increased following exposure to the conditioned stimulus (1, 2). Regardless of the precise nature of the behavioral response to stress, the current study found that cold stress demonstrated approximately 40% higher density of CB1-receptors in amygdaloid body compared with the control group. Besides, our previous experiments showed anxiogenic effect of cold stress in the open field test (5). The exact mechanism of cold stress due to endocannabinoid modulation of emotional behavior in rats is unclear. We may suggest the inhibition of wide range of neurotransmitters including glutamate, acetylcholine, norepinephrine, and GABA by endogenous ligands (ECBs) for the CB1 receptors. Endocannabinoids are released by an unknown mechanism and diffuse across the synapse to activate receptors located on the axon terminal, suppressing neuronal excitability and

inhibiting neurotransmission. They are inactivated rapidly by uptake (possibly carrier mediated) followed by intracellular hydrolysis. In general, the predominant effects of endocannabinoid signaling are to constrain activation of the HPA axis. (14, 15).

In conclusion, our findings revealed alterations in CB1 receptor density that occurs in response to cold exposure. May be this is one of the mechanisms by which cold stress affects the synaptic connectivity between amygdaloid nuclei and leads to modulation of emotional behavior, learning, and stress-response.

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