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Morphometric analysis of hypothalamic cells showing c-Fos proteins after movement restriction and EHF-irradiation

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Abstract

A morphometric analysis of hypothalamic cells containing c-Fos-like protein detected by an indirect immunoperoxidase method was carried out to clarify the responses to movement restriction and skin electromagnetic high frequency (EHF) irradiation (7.1 mm, 42.2 GHz, 20 mW output power) simultaneously to three acupuncture projection areas. The morphometry of c-Fos-protein positive neurons by their number and type was analyzed. Movement restriction (40 min) induced c-Fos protein expression primarily in cells with 10–50 μm^2 sizes (associative type neurons) only in anterior hypothalamic nucleus and lateral hypothalamic area; while additional EHF-irradiation of acupuncture projection areas (under movement restriction) induced c-Fos expression in all hypothalamic structures and mostly in cells with 70–150 μm^2 sizes (relay type neurons), i.e. changed the pattern of activated cell type distribution. In conclusion, the findings show that modest stress under which experimental animals often are exposed increase c-Fos protein expression in hypothalamic centers and skin EHF-irradiation of acupuncture projection areas seem to increase that.

Keywords: Immobilization; Electromagnetic high frequency (EHF) irradiation; Hypothalamus; c-Fos-positive cells; Relay associative type neurons; Acupuncture points (ST36 and DU14)

1. Introduction

The immediate response gene c-Fos is activated after exposure to different types of stressors [1,2]. For instance painful stimuli and e.g. an injection of antigen induce c-Fos protein expression in specific hypothalamic structures [3–5]. c-Fos protein positive cell counts depend upon the nature and intensity of the stressor [6]. Studies on the activity of the nervous system as judged by c-Fos gene expression are usually limited to the determination of the number of activated cells and their localization in different brain structures. The size determination of the cells expressing c-Fos genes would increase the information on the cell types recruited during the activation process and, indirectly, on their roles. Large amount of neurohistology data has been accumulated on the subcortical structures. Two basic types of neurons have been consistently distinguished in hypothalamic nuclei that differ in their size, architecture, and lengths of their dendrites and axons [7,8]. The organizational characteristics of axons and dendrites suggest that smaller-neurons may be associative (reticular) neurons, which are involved in the signal transmission within a specific structure (a nucleus or a field) while the larger neurons may be relay (projection) neurons. Their axons distribute signals beyond a given structure and are involved in communication between different hypothalamic structures, or may distribute even beyond the limits of the hypothalamus, i.e. they may be involved in the transmission of signals to far-a-way brain structures or to spinal cord.

The hypothalamic responses to movement restriction are practically unknown, although many experiments require the immobilization of the animal. Electromagnetic high frequency (EHF)-irradiation of the skin is also poorly known as far as the hypothalamic structures are concerned, although positive effects of EHF-irradiation of projections of the ST36 and DU14 acupuncture points have been observed during

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combined treatment of many diseases and even the toxicity of drugs [9–11]. EHF-irradiation of skin is a weak stressor, but it may have specific wave effects. Ranking of cells expressing c-Fos proteins according to their sizes helps to determine which cells are responding to stimuli and their roles.

The aims of the present study were to clarify the hypothalamic responses as judged by c-Fos protein expression after simple movement restriction and EHF-irradiation of certain acupuncture point projection areas in rat skin by using morphometric analyses of the activated cells.

2. Materials and methods

Experiments were carried out on 25 male Wistar rats weighing 180–220 g. Animals were maintained in accordance with standards for working with laboratory animals under usual vivarium conditions at room temperature with 12 h light/dark cycles. The rats had free access to food and water.

Animals were adapted to the experimental conditions by placing them in immobilizing containers for 40 min a day over a 5-day period. After adaptation during the experiment all rats were tranquil while staying in containers.

Experimental groups were (1) control 1—intact animals maintained under standard conditions in their cages. (2) Animals adapted to the experimental conditions and exposed to movement restriction in containers for 40 min. (3) Animals adapted to the experimental conditions, restricted in their movements and subjected and skin EHF-irradiation for 40 min before and 40 min after a period of 40 min of free motility.

EHF-irradiation. An electromagnetic field generator (model G4-141, Pushchino, Russia) was used as the radiation source with a 37–53 GHz frequency band. The penetration depth of EHF-irradiation at a 7.1 mm wavelength and 20 mW output power did not exceed 1 mm. Rat skin was irradiated at three points: both knees, 3 mm below and 3 mm lateral to the center of the knee-joint, and the skin of the posterior surface of the neck between the 7th cervical and the 1st thoracic vertebrae along the medial line (corresponding to projections of the ST36 and DU14 acupuncture points).

Brain fixation. After the experiments, rats were placed in standard cages and after 120 min, they were anesthetized by an i.p. phenobarbital dose (60 mg/kg body weight). Brain fixation was accomplished by intracardial perfusion with 100 ml of cold solution of 7.4 pH paraformaldehyde (4% paraformaldehyde in 0.1 M phosphate buffer solution pH 7.4 with 0.2% picric acid). The brain was cut free and fixed in a fresh batch of fixing mixture at +4 °C for 12 h and then embedded in paraffin.

Five-micrometer thick paraffin slices were cut. The slices were freed of paraffin with *o*-xylol at 60 °C for 30 min followed by rehydration with a series of increasingly diluted ethanol solutions. Parallely prepared thionine stained slices were used to characterize the topography of the brain and determine the total number of cells in a specific area of a slice. With the aid of standard Golgi silver staining and standard Nissl staining the morphology and branching of neurones were further elucidated.

Immunohistochemistry. c-Fos-like protein was detected by an indirect immunoperoxidase technique [12] using primary polyclonal antibodies against the family of c-Fos proteins (Santa Cruz Biotech Inc., CA, USA). The final concentration of c-Fos antibody was 1 μg/250 μl of 0.2 M PBS. Rabbit anti-IgG antibodies conjugated with peroxidase (Sigma, St. Louis, MO, USA) was used at a 1:300 dilution as secondary antibodies.

Cell counting and statistical analysis. The analysis of the activated hypothalamic cells was carried out in the following structures: the anterior hypothalamic area (AHA), supraoptic nucleus (SO), suprachiasmatic nucleus (SCH), arcuate nucleus (ARC), hypothalamic paraventricular nucleus (PVH), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), and the lateral hypothalamic area (LHA). Cells were counted using an Ista Video Test apparatus (St. Petersburg, Russia). Brain slices were examined by a Jenamed-2 light microscope. The Ista Video Test Master software was used to determine the size of cells and slices, the number of cells and the optical density of cell and background staining for each slice and structure. When counting of the number of c-Fos-positive cells, only cells stained at least 1.25 times above the background optical density were recorded as c-Fos-positive. The sizes of c-Fos-positive cells were determined by locating them in 4–6 sections passing through the central part of the analyzed structure. To compare the number of c-Fos-positive cells in different structures, averaged data of quantitative counts were recounted on a standardized size of 10,000 μm^2 for all analyzed structures.

To determine changes in the types of activated (c-Fos-positive) cells involved in a response to a particular stimulus, cells were ranked according to their sizes and divided into seven classes. Class 1 included cells having sizes ranging from 10 to 30 μm^2. (We cannot exclude the possibility that glial cells were also counted in this class.) Cells of the other classes were determined by 20 μm^2 size increments: class 2: 30.01–50 μm^2; class 3: 50.01–70 μm^2; class 4: 70.01–90 μm^2; class 5: 90.01–110 μm^2; class 6: 110.01–130 μm^2; and class 7: 130.01–150 μm^2.

Swanson's rat brain atlas [13] was used to characterize brain slices. The significance of differences was evaluated using the Student's *t*-test.

3. Results

In the intact control rats only few isolated c-Fos-positive neurons were revealed. In the experimental setup adapted animals with movement restriction, the counts of activated cells were 7.32–16.64% (Table 1) and increased numbers were seen in AHN and LHA compared with the control animals, but pattern of class distribution was not changed. c-

Table 1
Induction of c-Fos-like proteins in hypothalamic structures of Wistar rats after movement restriction and two additional EHF exposures

Experimental animals	% of c-Fos-positive cells relative to total cell counts per 10,000 μm^2 of slices of the specified hypothalamic structures						
	AHN	PVH	VMH	DMH	Basal LHA	Perifornical LHA	PH
Intact	9.42	5.89	7.08	11.19	7.47	8.65	7.25
After movement restriction	16.64*	7.32	7.88	12.23	14.26*	15.37*	7.37
After EHF-irradiation	52.74*,#	56.98*,#	45.52*,#	70.21*,#	60.10*,#	97.55*,#	67.61*,#

EHF-irradiation of skin was applied 40 min before and one 40 min after movement restriction.
* $P < 0.05$. vs. intact.
# $P < 0.05$. vs. movement restriction.

Fos-positive cells in the intact and control animals belonged only to the first two classes of cells, i.e. cells with sizes from 10 to 50 μm^2 (Fig. 1).

After movement restriction, the number of c-Fos-positive cells compared to the total number of cells varied from 7.32% to 16.64%. Because the density of the cell distribution is different in the basal and perifornical parts of the LHA, the cell counts were carried out for each part separately. The largest percentages of c-Fos-positive cells among the structures analyzed were found in the AHN, perifornical and basal parts of the LHA (Table 1). The number of c-Fos-positive cells increased mainly through the activation of cells in the first two classes of cells (from 10 to 50 μm^2). In addition to smaller-sized cells, middle-sized cells were also activated (Fig. 1).

In the experimental setup adapted and movement restricted rats the EHF-irradiation of the skin (corresponding to projections of the ST36 and DU14 acupuncture points) was

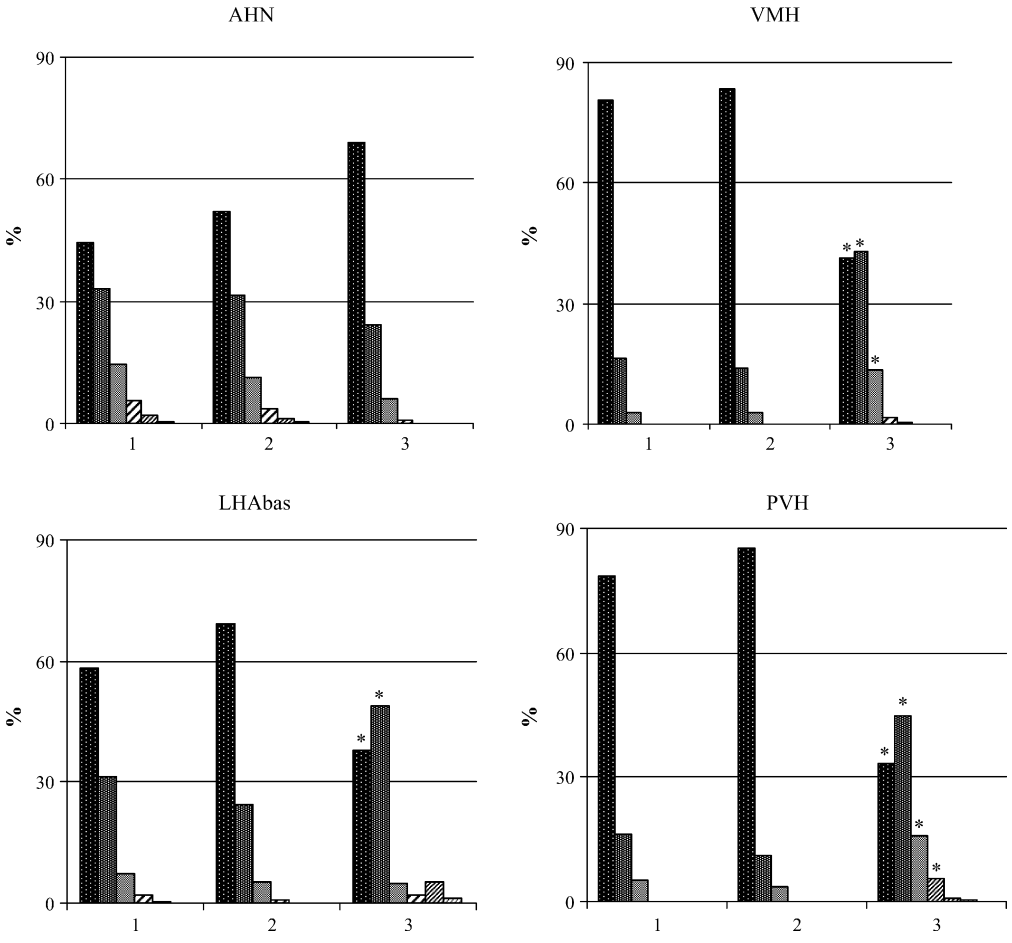

Fig. 1. Size-ranked distribution of c-Fos protein positive cells (% content of cells with different sizes (total number of c-Fos positive cells taken as 100%)) in hypothalamic structures of rats after 40 min movement restriction and additional skin EHF-irradiation in projections of the ST36 and DU14 acupuncture points. Sizes of c-Fos protein positive cells: (▨) 10–30 μM^2, (▤) 30–50 μM^2, (▨) 50–70 μM^2, (▩) 50–70 μM^2, (■) 70–90 μM^2, (▥) 90–110 μM^2, and (▦) 110–130 μM^2. Abscissa—experimental animals: 1—intact controls; 2—after movement restriction; 3—after further EHF-irradiation of skin in projections of the ST36 and DU14 acupuncture points. *$P < 0.05$ compared with the number of c-Fos-positive cells in rats after movement restriction.

associated with an increased cell activation in all hypothalamic structures. The percentage of c-Fos-positive cells was different in hypothalamic structures. The percent of activated cells was $97.55 \pm 2.4\%$ in the perifornical part of the LHA and $60.10 \pm 2.15\%$ in the basal part of the LHA (Table 1). The percent of c-Fos-positive cells in hypothalamic structures varied from $70.21 \pm 3.18\%$ to $45.52 \pm 2.39\%$. EHF-irradiation of the skin activated larger cells than the movement restriction only. The most pronounced changes in the distribution of smaller- and larger-sized cells were observed in the PVH, and VMH compared to the type of activated cells of animals after movement restriction.

A detailed analysis of the microcellular nuclei of the VMH was carried out as an example. The analysis of frontal sections using the Nissl method discriminated two groups of neurons that differed from each other in their sizes, nuclear-cytoplasmic ratio and localization in the VMH nucleus. Cells of the first group had diverse forms: fusiform, round, and triangular with 6–8 μm diameters. The nuclei of these cells primarily had oval or round shapes. Such neurons were encountered throughout the entire VMH nuclear volume. They were, however, more concentrated in its dorsal and ventral regions corresponding to the dorsomedial (DM) and ventrolateral (VL) parts of Swanson's rat brain maps [13].

In preparations treated with the Golgi silver stain method, the distinctive structures of these cells were revealed by their branching and orientation of processes, which are characteristic for these two groups. Cells of the first group typically had 2–4 main dendrites that could be traced up to 440 μm. The first branching of their dendrites occurred 20–30 μm from their cell bodies. The second-order dendrites become thinner as the distance from their cell bodies increased. They had spine-like protuberances over their entire length, whereas the third-order dendrites had a thread-like appearance with bead-like swellings (Fig. 2a). The neuron axons began at the soma or proximal part of the dendrite. The majority of these types of neurons showed that their axons sharply changed their direction at a small distance from their cell bodies, forming loops, dichotomically branching, or forming several collaterals within the limits of the branching dendrites (Fig. 2b). The presence of short collaterals terminating in synaptic boutons closely opposed to the dendrites of neighboring cells suggesting the possibility of axodendritic contacts between neurons of the first group.

Cells of the second group were characterized by their large sizes, 12–16 μm cell body diameters, polygonal shapes. The cell counts were significantly lower in the VMH than the cells of the first group. Processes of these cells had distinctive features. The number of their dendrites varied from 5 to 8 and their thickness changed little with distance from their cell bodies. At distal sites of 100–200 μm from the perikaryon, dendrites dichotomously branched and were supplied with spine-like processes over their entire lengths. Axons of these cells, as a rule, were distributed beyond the limits of their dendritic branchings and they possessed single collaterals.

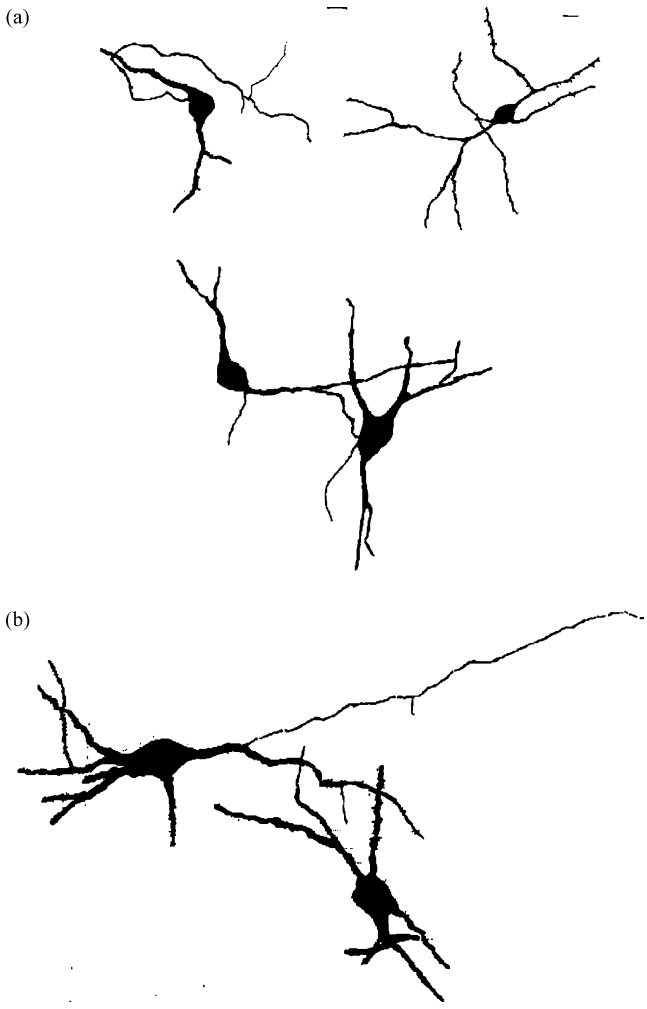

Fig. 2. Types of neurons in the VMH of the rat brain: (a) first type neurons (associative); (b) second type neurons (relay). Silver stain Golgi method. Scale bar—10 μm.

Cells of this group were similar to densely branched relay cells.

A comparison of the data obtained in this study suggests that c-Fos-positive cells having sizes of from 10 to 50 μm^2 were mainly associative type cells and that cells with sizes of from 70 to 150 μm^2 were mainly relay type cells.

4. Discussion

The present studies are the first to use morphometric methods to evaluate hypothalamic c-Fos-positive cells in the determination not only of the number but also the neuron types activated after exposures of the animals to different types of mild external stimuli (short movement restriction and mild skin EHF-irradiation of defined acupuncture projection areas). The activation of hypothalamic cells took place already by immobilization and even more after further electromagnetic high frequency (EHF)-irradiation.

The data comparison in morphometry of immunohistochemical preparations and descriptive characteristics of neurons revealed by the Golgi silver staining and also by the Nissl staining suggest that c-Fos-positive cells of sizes from 10 to 50 μm^2 relate mainly to cells of the associative type while cells with sizes from 70 to 150 μm^2 were of relay type. That refers to data of Millhouse [8]. Ranking of c-Fos-positive cells this way has not previously been done.

It has been shown that EHF-irradiation of skin leads to immunomodulating effect [14,15]. That could be mediated by stimulation of the activity of neuro-endocrine system [16]. To our knowledge our study is also the first of its kind where the EHF stimulation of defined acupuncture projection areas has been clarified at the hypothalamic level. These findings provide modestly perhaps some further understanding on the immunomodulation effects caused by acupuncture.

Measurement of the size of the activated cells showed that short term movement restriction, which is often necessary in common experimental studies, results in different degrees of activation of hypothalamic structures and causes an induction of c-Fos proteins mainly in cells of the associative type (10–50 μm^2).

Also EHF-irradiation of acupuncture points in the skin of movement restricted rats induced a further activation of cells in hypothalamus. The sizes of the activated cells were, however, substantially different from those seen after simple movement restriction: a significant part of the c-Fos-positive cells were larger and could be classified as cells of the relay type (70–150 μm^2). Unfortunately the present series of experiments with movement restriction and movement restriction with further irradiation were not completely comparable as the handling time in the last mentioned case was longer than in the first case.

The determination of primary targets of electro-magnetic stimuli was outside the scope of the present work and further studies are needed. One of the manifestations of any stimulus effects was an activation of the neurons in different structures of CNS. The c-Fos protein expression in neurons of these structures revealed after EHF-irradiation of skin evidence the response of CNS to this stimulus. The depth of the electro-magnetic wave penetration into the skin is known to be limited in 0.5–1 mm [16,17]. Thus, EHF-irradiation probably influenced only those cells that lied less deep than 1 mm under the skin surface. The skin is, however, significantly non-homogenic in projection sites of acupuncture points (the high density of mast cells, capillary network, free nerve terminals and Langerhance cells [18,19]) but no specific cells were observed [20].

The morphometric analysis of the activated cells showed that EHF-irradiation with movement restriction unlike movement restriction only induced activation of different types of neurons. Ranking of the activated cells according to their sizes showed that the effect of EHF-irradiation exposure was manifested not only in an increase of activated cell number but also changes the pattern of the activated neuron type distribution (a decrease of the associative and an increase of relay-type cells among the activated cells). After EHF-irradiation the distribution of activated cells according to their sizes was more significant in PVH.

As recently shown, the activation of even few neurons in cortex or brain stem influence the intensity of reactions. If this is true for the functioning of hypothalamic structures, it can partly explain the modulating effects of EHF-irradiation of skin on different functions and processes what has been shown by many authors [21–23].

The use of the morphometric method of analysis helped not only to determine the number of brain cells that were activated in response to the different types of stimuli, but also to determine the types of neurons that were activated. These results substantially increased our ability to scientifically map the specific types of brain cells involved in responses to different stimuli. Thus the morphometric method in the analysis of c-Fos-positive neurons may be helpful in acquiring additional information about the activated neurons.

One should note that the animals must be accustomed to the experimental condition, if the effects of different stimuli on the brain reactions are investigated, e.g. under movement restriction. After an adaptation the c-Fos-positive cell number in CNS was decreased compared with that in non-adapted animals, and it was the same as the c-Fos-positive cell number in intact animals. The number of activated cells in hypothalamic structures of non-adapted animals under the conditions of movement restriction has earlier been shown to be 5–20-fold higher than in intact animals [15].

In conclusion, the use of the morphometric analysis allowed not only to determine the number of hypothalamic cells that were activated in response to the different types of mild stimuli like movement restriction and skin stimulation, but also to determine the types of neurons that were activated. These results substantially increased our ability to map the specific types of hypothalamic cells involved in responses to specific stimuli. The analysis c-Fos-positive neurons provided additional information about the type of activated neurons. This activation seems to take place even in conditions like immobilization, which is rather common in animal experiments.

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