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C-fos gene expression induced in cells in specific hypothalamic structures by noxious mechanical stimulation and it's modification by exposure of the skin to extremely high frequency irradiation

Natalia S. Novikova[1], Tatiana B. Kazakova[1], Vernon J. Rogers[2] & Elena A. Korneva[1]

1. Department of General Pathology and Pathophysiology, Institute of Experimental Medicine, Russian Academy of Medical Sciences; RUSSIA.
2. Institute for Bio-Information Research, Wayne, Pennsylvania, USA.

Correspondence to: Korneva E.A. - Institute of Experimental Medicine, Russian Academy of Medical Sciences, Acad. Pavlov Str. 12, Saint Petersburg 197376, RUSSIA
TEL: + 812 234 07 24, FAX: +812 234 94 93,
E-MAIL: korneva@VK5270.spb.edu

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ORIGINAL ARTICLE

Abstract

OBJECTIVES: The purpose of this study was to determine: 1. The spatial pattern of c-fos gene expression in rat hypothalamic neurons after exposure to NMS and 2. The expression of c-fos gene after combined exposure to NMS and EHF irradiation of the rat skin. **METHODS:** The experiments were performed on 28 male adult Sprague-Dawley rats. After rats were subjected to noxious mechanical stimulation (NMS) or its combination with EHF irradiation of the skin, cells in various hypothalamic structures were analyzed to determine theirs effects on c-fos gene expression, an accepted marker of the activation of neurons. C-Fos-like protein was revealed by an indirect immunoperoxidase method. **RESULTS:** This study revealed that NMS stimulates c-fos gene expression in the anterior hypothalamic nucleus (AHN), dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH), and lateral hypothalamic area (LHA) by 157, 101, 199 and 115% respectively compared to control animals. Combined exposure to NMS and EHF irradiation directed at the skin in the region of the St36 acupuncture point projection site (left side) results in a decrease of the number of neurons that are activared in the AHN, DMH, VMH, and LHA by 33.6, 13.2, 31.0 and 32.9% respectively compared to the number of neurons activated by exposure to NMS by itself. EHF exposure of the skin of rats not subjected to NMS differentially effects the number of c-Fos positive cells expressed in hypothalamic structures: a decrease of 39.7% was observed in the number of activated neurons in the central part of LHA (level 28) compared to sham-irradiated animals, while an increase of 80.95% was noted in the number of c-Fos positive cells in the DMH compared to sham-irradiated animals.

CONCLUSIONS: The spatial pattern and degree of activation of c-fos gene expression has been shown in cells of the hypothalamus of rats after exposure to NMS by itself and after NMS combined with EHF irradiation of the skin. The "stress" reaction of cells in specific hypothalamic struc-

tures has been shown to be decreased after EHF exposure of the skin.

ABBREVIATIONS

AHN – anterior hypothalamic nucleus
DMH – dorsomedial hypothalamic nucleus
VMH – ventramedial hypothalamic nucleus
LHA – lateral hypothalamic area
SCH – suprachiasmatic hypothalamic nucleus
SO – supraoptic hypothalamic nucleus
PVH – paraventricular nucleus hypothalamus
Arc – arcuate hypothalamic nucleus
NMS – noxious mechanical stimulation
EHF – extremely high frequency
McH – melanin-concentrating hormone
ORX – orexin

Introduction

It is known that expression of the immediate early response gene, c-fos, an accepted marker of the activation of neurons, permits detection of both the brain structures as well as the specific cells within those structures that participate in the realization of reactions to specific stimuli. It has been shown that, after exposure to different stimuli, c-fos gene expression occurs in different brain structures, including those in the hypothalamus [1–3]. There are no studies in the literature in which an analysis of c-Fos positive neurons was performed for the purpose of revealing the possible effects of other stimuli on the reactions of hypothalamic neurons to noxious stimuli. Interest in hypothalamic structures stems from the fact that the hypothalamus is the most important formation participating in the regulation of visceral functions, sensory processes, hormonal balance and in the realization of neuroendocrine system reactions to any stimuli of a stressful nature [4]. The number and spatial pattern of c-Fos positive hypothalamic neurons may differ as a function of the nature of the stimulus [1,3,5], which may make it possible to determine the extent of the effect of different types of external stimuli on hypothalamic functions. EHF radiation has been used in animal studies and in medical practice for the treatment of patients with different diseases, including pain syndromes [6–8]. Combined exposure to two or more stimuli may result in a summation or, on the contrary, a reduction of their effects on c-fos gene expression.

The goals of this study were to determine 1. The spatial pattern of c-fos gene expression in rat hypothalamic neurons after exposure to NMS and 2. The expression of c-fos gene after combined exposure to NMS and EHF irradiation of the rat skin.

Materials and methods

The experiments were performed on male adult Sprague-Dawley rats weighing 180–200 g. Animals were housed in cages at room temperature on a 12h light-dark cycle (8 a.m.–8 p.m.) with free access to food and water. Rats were taken for experimentation at the same time of day (11–12 a.m.). Except for intact group (1st group), all groups of animals (2nd–7th groups) were under initial (1.5%) halothane narcosis before the experiments. Animals were narcotized by placing and keeping them in an inhalation chamber until the appearance of the first signs of musculature relaxation. They were then removed from the chamber and immobilized by suspending them in cloth hammocks. This study was carried out in accordance with the ethical guidelines described in the European convention directives 86/609 ESC for the care and use of experimental animals.

Experimental groups

A total of 28 animals divided into seven groups (four animals in each group) were used in this study:

1. intact group (1st group). (Animals kept in home cages under standard laboratory conditions and not subjected to any additional treatments)
2. control group (2nd group) (animals restrained in cloth hammocks under initial halothane narcosis)
3. stressed group (3rd group) (animals restrained in cloth hammocks under initial halothane narcosis and subjected to NMS)
4. sham-irradiated group (4th group) (animals restrained in cloth hammocks under initial halothane narcosis and exposed to sham-irradiation)
5. sham-irradiated/NMS group (5th group) (animals restrained in cloth hammocks under initial halothane narcosis and subjected to sham-irradiation and NMS)
6. irradiated group (6th group) (animals restrained in cloth hammocks under initial halothane narcosis and subjected to a single 45 min EHF exposure)
7. irradiated/NMS group (7th group) (animals restrained in cloth hammocks under initial halothane narcosis and subjected to NMS and EHF exposure, one 20 min exposure before NMS and one 25 min exposure beginning right after NMS).

Experimental stress model

Noxious mechanical stimulation was used as the model of experimental stress. It was administered by compressing the rat's hindpaw with a surgical clamp 10 times for 10 sec over a 10 min period [4].

EHF irradiation of the skin

A G4-141 generator (Pushchino, Russia) with a 10 mW output power and 42.19 GHz (7.1 mm) frequency served as the EHF radiation source. Selection of EHF irradiation parameters and their measurements were in accordance with international standards [7, 8, 9]. The generator output was connected to a dielectric waveguide that terminated in a 20 mm diameter floroplastic applicator which was secured at a distance of 1 mm from the surface of the rat's skin 3 mm below and 3 mm lateral to the center of the left knee, corresponding to the St36 acupuncture projection site. This area of the skin was selected for EHF exposure based upon data in the literature indicating that, in both humans and animals, weak electrostimulation of the St36 acupuncture point decreases the reaction of neurons in the brain to a painful stimulus [10].

Fixation of the brain and treatment of sections

After completion of experiments, animals were placed in their hame cages and narcotized with phenobarbital (60 mg/kg) 120 min later. Intracardial perfusion was then performed with a warm physiologic solution with heparin (10 u/ml, 100 ml, 10–15 ml/min) and then with a cooled fixing solution [100 ml 4% paraformaldehyde per 0.1 M PBS and 0.2% picric acid (pH 7.4)]. An hour later the perfused brain was removed and additionally fixed in a new batch of fixing mixture at 4°C for 12 hours, followed by embedding in paraffin. 5 µm thick paraffin sections were mounted on slides. Sections were deparafinized at 60°C in orthoxylene for 30 minutes followed by rehydration in a reducing gradient of ethanol to water.

C-Fos-like protein

C-Fos-like protein was revealed by an indirect immunoperoxidase method using primary polyclonal antibodies against the family of c-Fos proteins (Santa-Crus, Biotech.Inc.). The final concentration of c-Fos antibodies was 1 µg per 250 µl 0.2M PBS. Antirabbit IgG labeled with peroxidase (Sigma) diluted 1:300 was used as secondary antibodies. Sections used for control were prepared for: 1) histological staining (characterization of brain topography), 2) immunohistochemical staining, a – control for endogenic peroxidase and b – control for specificity of binding of secondary antibody (without addition of primary antibodies).

Count of cells and statistical analysis

Count of labeled cells was made in the monitor frame (12 cm x 12.5 cm, with 1 cm=25 µm) using the Ista-Vidio-Test (St. Petersburg). An average of 6 sections was analyzed for each structure of each animal. Swanson's stereotaxic atlas of the rat brain [11] was used to characterize the structure of a brain section. Data are presented as the means ± SEM. Comparisons between groups were made using one-way analysis of variance (ANDVA). Comparisons between two groups were made using Student's *t* test. Differences with p values of 0.05 were considered significant.

Results

An analysis of the distribution of the number of c-Fos positive cells resulting from different stimuli have revealed that isolated c-Fos positive neurons are encountered in hypothalamic structures of intact animals. After initial halothane narcosis was established and animals immobilized in cloth hammocks, the number c-Fos activated cells increased by 5–10 times in the anterior hypothalamic area (AHA), supraoptic nucleus (SO), suprachiasmatic nucleus (SCH), paraventricular hypothalamic nucleus (PVN), arcuate nucleus (Arc), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH) and lateral hypothalamic area (LHA) of control animals (2nd group) compared to intact animals (1st group). The number of c-Fos positive cells did not change in the SCH, SO, PVH and Arc of animals subjected to NMS (3rd group) compared to control animals (2nd group). However, marked changes of the number of c-Fos positive cells were noted in the AHN, VMH, DMH and LHA of these animals (3rd group) compared to control animals (2nd group) (Table 1). The percentage of increase of immunoreactive cells in these nuclei and area was 157, 199, 101 and 115% respectively compared to control animals (2nd group). The most intense reaction to NMS was noted in the VMH nucleus (199%). It must be emphasized that the average number of c-Fos positive cells throughout the entire extent of the tuberal part of the lateral hypothalamic area does not completely reflect the character of

Table I. C-Fos–like protein expression induced by noxious mechanical stimulation (NMS) and EHF irradiation of the skin in cells of specific hypothalamic structures of Sprague-Dawley rats.

		Number of c-Fos immunoreactive cells in hypothalamic structures Hypothalamic structures.					
		AHN	**VMH**	**DMH**	**LHA**	**LHA**	**LHA**
		Section levels according to Swanson'a rat brain map					
Groups of rats		**25-27**	**27-29**	**28-30**	**27**	**28-29**	**30-32**
Intact	n=4	3.5±0.27	2.33±0.41	1.11±0.57	2.3±0.41	2.60±0.54	1.11±0.57
Control	n=4	17.0±2.34 ∇	17.0±2.34 ∇	20.0±2.54 ∇	17.03±2.34 ∇	17.0±1.56 ∇	7.05±2.34 ∇
Given NMS	n=4	43.7±2.85 ∇	50.8±4.11 ∇	40.2±4.00 ∇	33.2±2.51 ∇	42.16±2.23 ∇	13.1±2.00 ∇
Given sham irradiation	n=4	17.0±3.28 ∇	18.0±1.34 ∇	21.0±2.54 ∇	17.8±2.34 ∇	20.2±1.56 ∇	14.1±2.51 ∇
Given sham irradiation and NMS	n=4	44.1±3.35 ∇	52.9±4.11 ∇	39.5±4.16 ∇	33.6±2.1∇	42.16±3.83 ∇	11.0±2.32 ∇
Given EHF irradiation	n=4	18.0±1.64 ∇	20.5±1.64 ∇	38.0±2.34 ∇♦	15.3±1.52 ∇	12.18±1.34 ∇♦	11.0±2.32 ∇
Given EHF irradiation and NMS	n=4	29.3±2.5 ∇*	36.5±2.34 ∇*	34.3±3.1 ∇	25.2±1.50 ∇*	32.02±1.34 ∇*	2.2±1.41 ∇*

P<0,05: ∇ – in comparison with the number of c-Fos positive cells in intact rats;
P<0,05: – in comparison with the number of c-Fos positive cells in control rats;
P<0,05: * – in comparison with the number of c-Fos positive cells in sham irradiated rats under NMS;
P<0.05: ♦ – in comparison with the number of c-Fos positive cells in sham irradiated rats;
n – the number of rats in experiments; Statistical analysis was made by *Student's t-test*

the reactions of cells in this area of the hypothalamus to NMS. A differential count of immunopositive cells in the LHA (levels 27–32 according to Swanson's atlas [11]) established in specific frontal sections of the brain that after NMS the maximum expression of c-Fos-protein (148%) was found in the LHA at levels 28–29 and the minimum expression (87%) was found in a more caudally located part of the LHA (levels 30–32).

It was of interest to see if it would be possible to modify the intensity of NMS-induced c-fos gene expression in hypothalamic structures by combining it with EHF irradiation of the skin. It turned out that EHF irradiation of the skin combined with NMS (7th group) did not result in any changes in the number of c-Fos-positive cells in the SO, SCH, PVH and Arc nuclei compared to the number in the experimental animals of the sham-irradiated/NMS group (5th group). At the same time, the effect of combined exposure of the animals to NMS and EHF irradiation of the skin (7th group) revealed itself in a decrease of the number of c-Fos positive cells in the AHN, DMH, VMH, and LHA by 33.6, 13.2, 31.0 and 32.9% respectively compared to the number in the 5th group of animals. The most pronounced decrease in the number of c-Fos positive cells (44.2%) was observed in the caudally located part of the LHA (levels 30–32). Photomicrographs of frontal sections of brain (VMH) from rats of the different experimental groups are presented in *Fig 1* as examples of changes in c-Fos-like protein expression in hypothalamic cells after different variations of exposure.

An analysis of hypothalamic structures after EHF irradiation of the skin (6th group) as an imdependent stimulus revealed changes in c-Fos protein expression in only two hypothalamic structures: a 39.7% change was observed in the number of activated neurons in the central part of the tuberal region of the LHA (level 28) compared to sham-irradiated animals (4th group), while an 80.95% increase was recorded in the number of c-Fos-positive cells in the DMH compared to the same group of animals.

Discussion

An analysis of c-fos gene expression, an accepted marker of the activation of neurons, after exposure to different types of external stimuli has revealed a differential degree of activation of neurons in a number of hypothalamic structures. The procedure used to prepare animals for experimentation (induction of initial halothane narcosis and suspension in cloth hammocks) was a very intense stressor that resulted in a sharp increase in the number of c-Fos positive cells expressed in SO, SCH, PVH, and Arc nuclei of the animals. NMS applied against the background of narcosis and immobilization failed to alter the number of activated cells in these nuclei. However, this result should be tested in an experimental model that uses adapted rather than intensely restrained animals. A determination of the spatial pattern of neurons activated in hypothalamic structures after application of NMS revealed a significant increase in the number of c-Fos-positive cells in the ANH, DMH, VMH and LHA. The significant modifying effect of EHF irradiation against the background of NMS was observed only in specific hypothalamic structures (AHN, VMH and LHA) and was expressed in a decrease in the number of activated cells. EHF exposure by itself (without NMS; 6th group) resulted in changes only in the DMH and LHA: 1). The number of c-Fos-positive cells decreased by 39.7% in the central part of the tuberal region of the LHA (level 28) compared to sham-irradiated animals (4th group) and 2). The number of c-Fos-positive cells increased by 80.95% in the DMH compared to sham-irradiated animals (4th group).

The differences discovered in the numbers of cells activated within the same structure (LHA) in response to NMS again demonstrates the morphofunctional heterogeneity of this area. It is interesting that the most pronounced reaction was noted in the central part of the tuberal region of the lateral hypothalamic area, which includes the perifornical area at the tuberal level of the LHA. There are many works available in the current literature dedicated to the study of neuropeptides and their locations that have shown that a relatively small number of orexin-ORX-containing neurons concentrated in the so called perifornical nucleus of the hypothalamus have projections to many different brain structures [12]. Neurons containing melanin concentrating hormone (McH) [13, 14] have also been shown in the perifornical region of the tuberal lateral hypothalamic area. Both of these peptides appear to be involved in reactions to stress [14]. Receptors for these neuropeptides have been demonstrated in many areas of the brain, including the VMH and DMH [15]. It is possible that the changes in the number of activated neurons that occurred in response to NMS was brought about by activation of ORX – and/or MCH – containing neurons in the tuberal part of the LHA and, possibly, by a modulation of the activity of neurons in other hypothalamic structures after interaction of ORX- and/or MCH with their receptors in these structures. It is suggested that hypocretin neurons of the tuberal part of the LHA downregulate the reaction to pain [16]. Double immunohistochemical staining for c-Fos and orexin make it possible to determine the role of orexin-containing neurons in the realization of the effects of EHF irradiation on the stress reaction to pain [17]. However, further studies are required to confirm these possibilities.

It should be noted that combined exposure to NMS and EHF radiation decreased the effect of NMS i.e., the activation of c-fos gene expression, with the largest decrease in the number of activated cells being noted in the VMH and caudal part of the LHA. However, EHF irradiation of immobilized animals (6th group) did change the extent of cells activated in hypothalamic structures only in the DMH and LHA. It is of interest that the number of activated cells increased in the DMH while it decreased in the central part of the tuberal region of the LHA. These results indicate the occurrence of changes in the extent of activation of cells in specific hypothalamic structures in response to EHF irradiation of the skin and are in agreement with the effects of EHF therapy used in the treatment

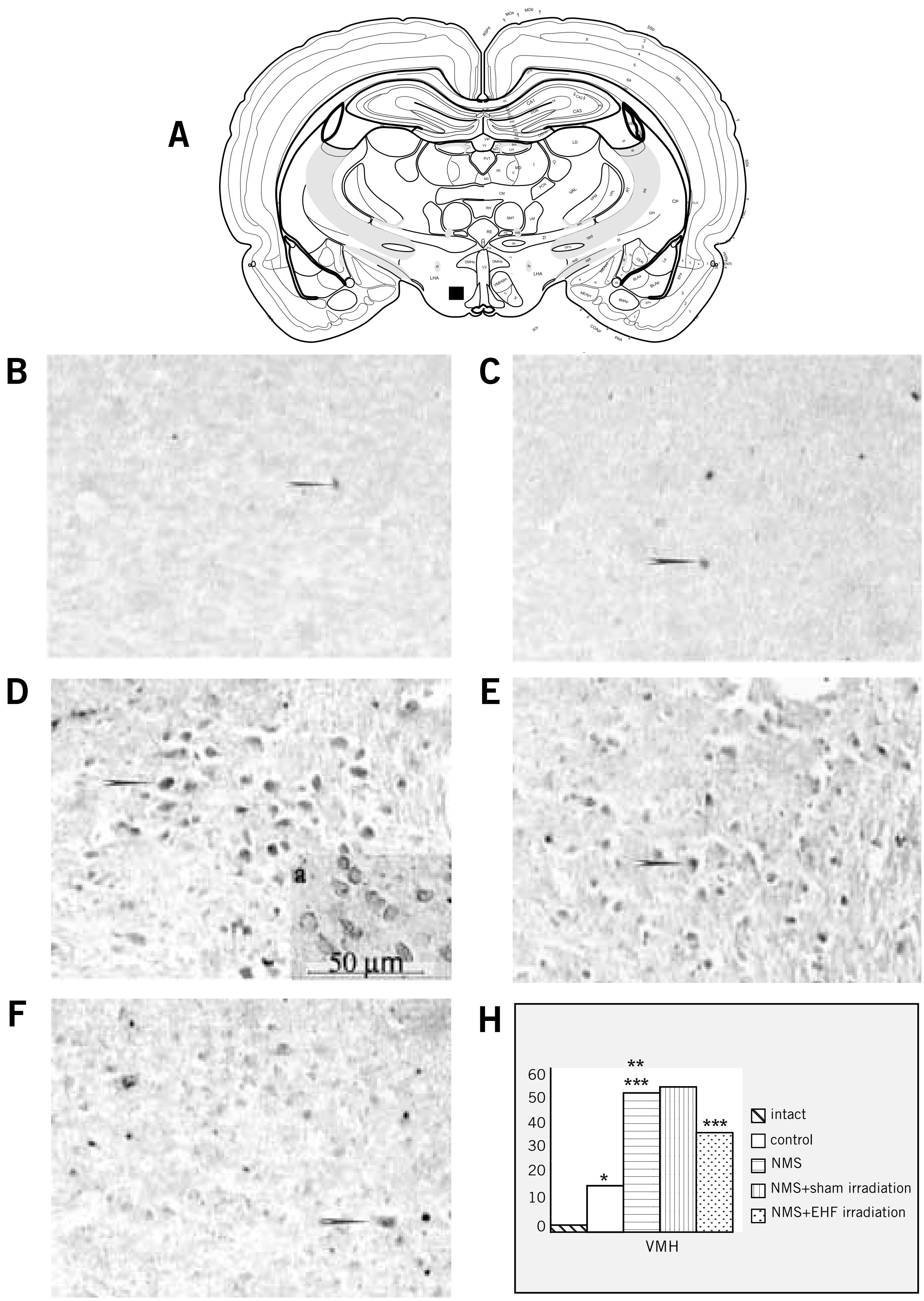

Figure 1. Photomicrograph of the rat brain VMH sections with c-Fos positive cells.
A – scheme of the rat brain (level 28 of the brain section according to Swanson's rat brain maps). ■ – analyzed VMH structure;
B – G – immunohistochemical staining x 10; **a** – x40. Animals: **B** – intact; **C** – control fixated in the hammock; **D** – after a NMS application; **E** – after sham EHF irradiation of the skin under NMS; **F** – after EHF irradiation under NMS; H – the average number of c-Fos positive cells in the VMH sections under different stimuli. Ordinate: the number of c-Fos positive cells.
P<0.05 – * – in comparison with intact rats; **P<0.01** – ** – in comparison with intact rats; **P<0.05** -*** – in comparison with control rats; **P<0.05** – **** – in comparison with NMS; in comparison with sham irradiated rats under NMS.

of patients with different diseases, including pain syndromes [6, 7].

This study showed for the first time:

1) The spatial pattern and degree of activation of c-fos gene expression in rat hypothalamic cells after exposure to NMS and
2) A decrease of the reactions of cells of specific hypothalamic structures resulting from combined exposure to NMS and EHF radiation. Different types of external stimuli elicit differential activation responses in different hypothalamic structures. Detection of c-Fos-like protein in brain cells makes it possible not only to determine the effect of exposure to different types of stimuli but also to determine the modulatory effect of other stimuli combined with them. For example, it may be used to analyze the effects of drugs that can only be observed against the background of some dysfunction

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