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A Blocker of NO Synthase Intensifies *c-fos* Expression in Spinal Neurons of Rats Realizing Stereotypic Movements

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Neurons with the presence of c-Fos protein, the product of expression of the "early' *c-fos* gene, were detected in the cervical region of the spinal cord of rats performing stereotypic operant food-procuring movements by the forelimb. An immunohistochemical technique was used; the presence of c-Fos was interpreted as a correlate of the activated state of the respective neurons. Effects of suppression of the activity of neuronal NO synthase (nNOS) provided by systemic injections of a selective blocker of this enzyme, 7-nitroindazole (7-NI), were studied under the above conditions. In the absence of 7-NI, the main foci of Fos-immunoreactivity in the spinal segments C6 and C7 were observed in laminae 2i, 3, and 4 of the gray substance, while after injection of this blocker these were found in laminae 3, 4, and 6 and also in the motor nuclei (lamina 9). In animals that performed repetitive food-procuring movements and were preliminarily injected with 7-NI, significantly greater mean numbers of Fos-immunoreactive neurons were found in slices of the gray matter, as compared with the respective figures in rats with no suppression of nNOS activity (54.4 ± 0.7 cells in a 40 μm-thick slice ipsilaterally with respect to the working limb, as compared with 31.7 ± 1.1 cells, $P < 0.05$). Thus, expression of the *c-fos* gene in certain regions of the cervical part of the spinal cord increases significantly under conditions of 7-NI-induced suppression of NO production; this is observed in rats realizing repeated movements for a long time.

Keywords: protein c-Fos, immunoreactivity, neuronal NO synthase (nNOS), 7-nitroindazole (7-NI), spinal cord, operant food-procuring movements.

INTRODUCTION

Sensory influences from muscle proprioceptors play a very important role in the functioning of spinal motor centers. Excitatory and inhibitory interneurons of the dorsal and ventral horns of the spinal cord, activated by these influences and by influences coming from suprasegmental centers, are components of the complex neuronal networks involved in the control of motor activity. In rats, descending projections from the sensorimotor cortex and various brainstem centers are known to spread down to layers 4-6 of the gray matter and also to dorsal and ventramedial parts of the intermediate zone (lamina 7) [1]. Such descending projections can modulate the activity of spinal interneurons, which is a key factor in the control of motor activity in animals. Neuronal activities in the brain and spinal cord of animals, which is related to realization of different movements of the natural motor repertoire, were studied in a few experimental studies [2-4]. It should, however, be recognized that information on changes in the interneuronal and motoneuronal activity in the spinal cord in the course of performance of motivated stereotypic movements is still obviously insufficient for adequate interpretation of the mechanisms controlling such motor phenomena.

At present, detection of a product of expression of the "early" gene *c-fos*, c-Fos protein, is extensively used for identification of activated neuronal populations in one structure of the CNS or another. Earlier, changes in the spatial distribution of Fos-immunoreactive (Fos-ir) neurons in the rat spinal cord were observed after locomotion on a treadmill. Activated neurons were found in the gray matter of the cervical and lumbar segments in the layers where interneurons responding to non-nociceptive stimulation are localized [2, 5]. In our recent experiments, we found changes in the level of *c-fos* expression (marker of neuronal activity) in the rat spinal cord after repeated realization of operant food-procuring movements of the forelimb. Neuronal activity under these conditions was rather

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similar to that observed after non-nociceptive stimulation of segmental afferent inputs, while the number and spatial distribution of activated cells in the gray matter layers was somewhat different [4].

As is known, the nitrergic system providing synthesis of nitric oxide (NO) is an important modulatory system in the spinal cord. Localization of neurons containing neuronal NO synthase (nNOS) in spinal structures is one of the most important characteristics of spinal centers involved in the control of one type of activity or another [6, 7]. As was shown, some NOS-containing cells are also GABA- and glycinergic neurons, i.e., inhibitory interneurons [8, 9]. Production of the volume mediator NO by such neurons influences NMDA-mediated facilitation of synaptic transmission, mechanisms of central sensitization, and those of spinal hyperalgesia [10]. As is supposed, modulation of the level of NO production should, in some way, affect the activity of neuronal networks involved in the control of motor phenomena. Isolated systemic introduction of an nNOS blocker, 7-nitroindazole (7-NI), was found to induce no significant changes in Fos-immunoreactivity in the rat spinal cord. Recently, however, we found that introduction of this blocker in rats subsequent vibrational stimulation of the tendon of the *triceps surae* results in increase in the level of Fos-immunoreactivity in motoneurons and interneurons in the lumbar spinal segments [11].

In the experiments described below, we examined peculiarities of the spatial and quantitative distribution of moto- and interneurons that are activated in the cervical spinal segments of rats after repeated realizations of food-procuring movements by these animals in the norm and after suppression of nNOS activity by its selective blocker (7-NI).

METHODS

Male Wistar rats (body mass 240-290 g) were used in the experiments. Animals were divided into five groups. Intact animals formed group 1; rats i.p. injected with a solvent of 7-NI, dimethyl sulfoxide (DMSO), formed group 2; group 3 included rats i.p. injected with 7-NI dissolved in DMSO. In group 4, animals performed repeated stereotypic food-procuring movements by the forelimb, while rats of group 5 performed such food-procuring movements after preliminary injections of 7-NI. In all groups, the number of animals $n = 4$. Rats of group 3 and 5 were injected with 30 mg/kg 7-NI (Sigma, USA) dissolved in 1 ml DMSO; the obtained solution was diluted by distilled water to a final concentration of DMSO of 4%. Under such conditions, there were no significant changes in the mean arterial pressure during maximum suppression of the NOS activity resulting from 7-NI injections [12, 13].

Animals of groups 4 and 5 performed, after 24-h-long food deprivation, repeated capturing of food globulae by the forelimb from a manger. Twelve everyday sessions with realization of such movements were carried out; the duration of the sessions was 30 to 40 min.

Two hours after termination of each experiment, rats were perfused intracardially by a fixative via the ascending aorta, perfusion was performed under deep anesthesia (sodium pentobarbital, Sigma, USA,

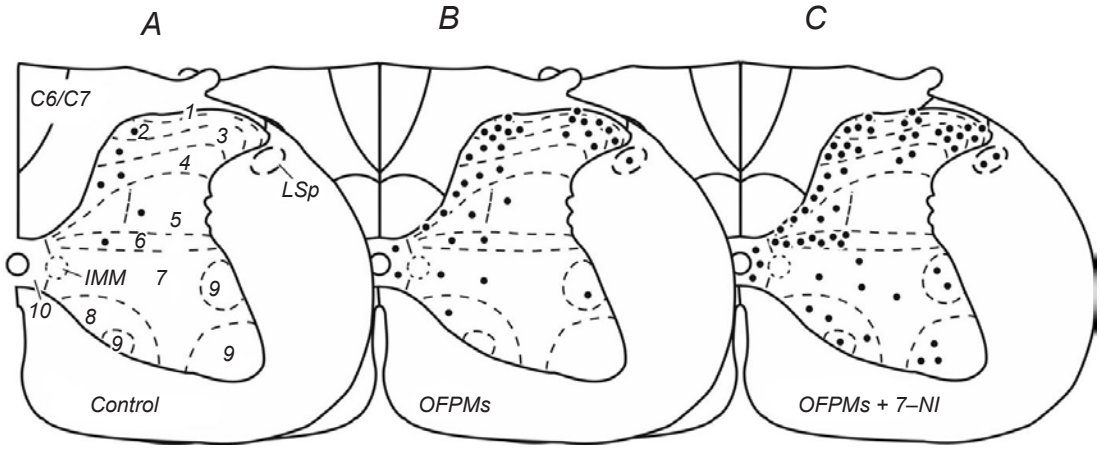

Fig. 1. Spatial distribution of Fos-immunoreactive neurons in spinal cord slices of control rats (A), rats realizing operant food-procuring movements (OFPMs, group 4) (B), and rats performing such movements under conditions of blocking of neuronal NO synthase by 7-nitroindazole (OFPMs + NI, group 5) (C). 1–10 are laminae of the gray matter, IMM is the intermediomedial nucleus, and LSp is the lateral spinal nucleus. In B and C, halves of the slices ipsilateral with respect to the working limb are shown.

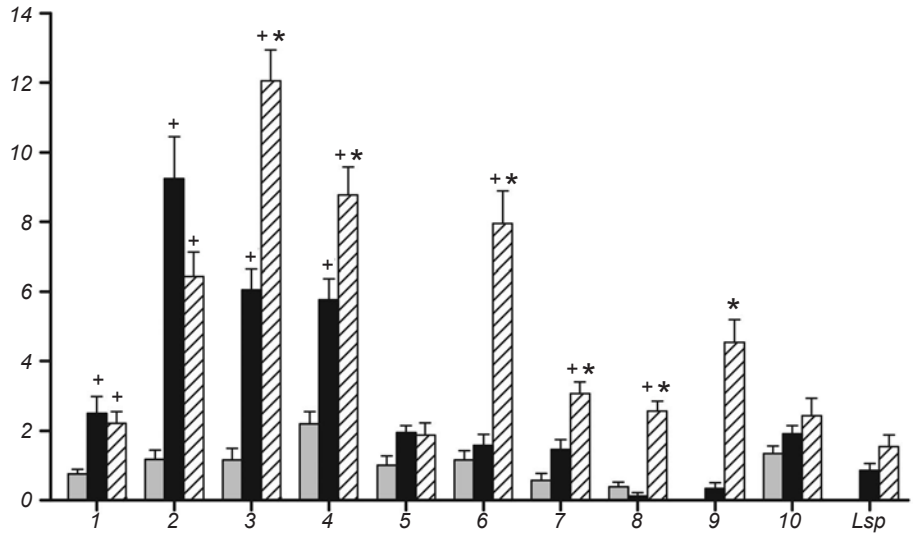

Fig. 2. Numbers of Fos-immunoreactive (Fos-ir) neurons in different laminae (1-10) of the gray matter of spinal segments C6 and C7 of rats of the control group 1, rats after realization of operant food-procuring movements (group 4), and rats performing such movements after blocking of neuronal NO synthase by 7-nitroindazole (group 5); gray, filled, and dashed columns, respectively. Mean numbers of Fos-ir units within half of the spinal cord slice (40 μm thick) of group-1 rats and halves ipsilateral with respect to the working limb of group-4 and group-5 rats are shown. Crosses designate cases of significant intergroup differences at comparisons of groups 4 and 5 with the control group 1, asterisks show analogous cases where group 5 was compared with group 4 ($P < 0.05$).

90 mg/kg, i.p.). Lower cervical segments of the spinal cord (C6 and C7) of each animal were cut off, additionally fixed, and immersed in a 30% sucrose solution, to provide cryoprotection. Cross-sections of the spinal cord (40 μm thick, 10-15 sections from each animal) were prepared on a freezing microtome for subsequent immunohystochemical staining. Detection of Fos-ir neurons in the sections was done using a standard avidin-biotin-peroxidase technique [14]; nuclei of such neurons were stained dark brown (Fig. 3). We calculated the mean number (± s.e.m.) in slices of the spinal C6 and C7 segments unilaterally at the left in rats of groups 1–3 and ipsilaterally with respect to the left working limb in slices of animals of the remaining two groups. The mean numbers of labeled cells were compared using two-parameter dispersion analysis of variations (ANOVA). In the cases where significant intergroup differences ($P < 0.05$) were found, the *aposterior* Newman–Keuls criterion was used.

RESULTS AND DISCUSSION

A relatively small number of Fos-ir neurons (5 to 10 units per slice unilaterally) was found in control animals (both intact ones and those injected with DMSO or/and 7-NI). Labeled neurons were observed in different laminae of the gray matter. It should be mentioned that Fos-ir neurons were absent in the motor nuclei (lamina 9) and lateral spinal nucleus (LSp) of animals of these groups (Fig. 1 A); there were no significant differences between the numbers of labeled neurons in left and right halves of the gray matter in animals of groups 1-3.

After realization of the motor program (operant reflexes) by the rats of group 4, a significantly greater mean number of labeled Fos-ir neurons ($P < 0.05$) was observed ipsilaterally to the working limb in segments C6 and C7, as compared with the respective number in control animals. Labeled neurons were most numerous within layers 2i, 3, and 4 of this side (9.2 ± 1.2, 6.1 ± 0.5, and 5.8 ± ± 0.6 units per slice, respectively (Fig. 1 B, 2). Small numbers of labeled cells were also localized within laminae 5-10 both ipsi- and contralaterally. In motor nuclei, single Fos-ir motoneurons were found; in the LSp, also there were single labeled units. It should specially be emphasized that the general intensity of *c-fos* expression was significantly higher in the examined spinal segments of rats that realized stereotypic food-procuring movements after preliminary 7-NI injections (group 5), as compared with that in animals of group 4 (54.4 ± 0.7 vs. 31.7 ± 1.1 units; $P < 0.05$) (Fig. 1 C; 2). Main foci of Fos-immunoreactivity in rats of group 5 were localized ipsilaterally in laminae 3. 4, and 6, and also in the motor nuclei (layer 9) (Fig. 3). The mean

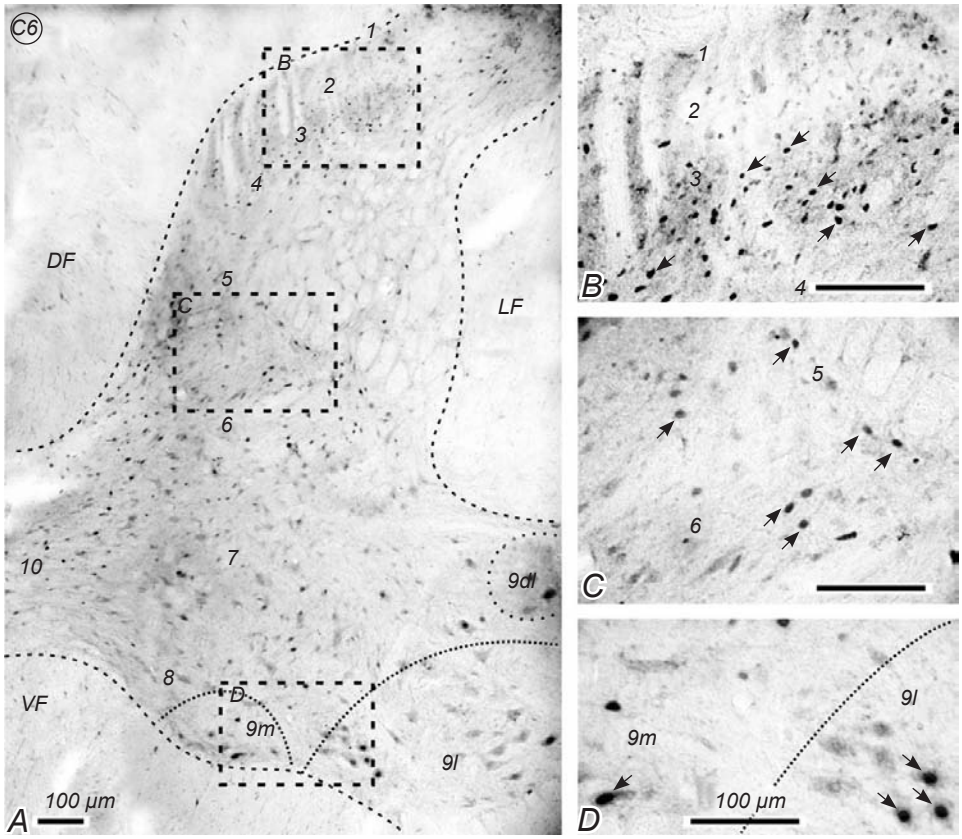

Fig. 3. Photomicrographs of Fos-immunoreactive (Fos-ir) neurons in a slice of segment C6 of the spinal cord of a rat after realization of operant food-procuring movements under conditions of blocking of NO synthase by 7-nitroindazole. Zones of localization of labeled neurons bounded by dashed lines are shown in B-D at a greater magnification. Black arrows indicate Fos-ir neurons in B and C and Fos-ir motoneurons in D. 1–10 are laminae of the gray matter; 9l, 9m, and 9dl are the lateral, medial, and dorsalateral parts of lamina 9; DF, VF, and LF are the dorsal, ventral, and lateral funicles of the white matter. Calibration 100 μm is general to all panels.

numbers of labeled neurons in the above-mentioned laminae were 12.1 ± 0.9, 8.8 ± 0.8, and 7.9 ± 0.9 units in layers 3, 4, and 6, respectively; in layer 9, the respective index reached 4.7 ± 0.7. It can be noted that the mean number of activated motoneurons in the medial, lateral, and dorsolateral groups of motor cells was rather close to each other. It should also be mentioned that the pattern of laminar distribution of Fos-ir neurons in the spinal segments C6 and C7 in the animal group 5 demonstrated no dramatic differences from the type of spatial distribution of labeled neurons in these segments of group-4 rats; these distributions differed from each other only quantitatively ($P < 0.05$).

Activation of early genes is known to play a significant role in the development of plastic processes in both motor cortex and spinal cord [15]. Expression of the early genes, such as *c-fos*, is an important trigger factor for the development of short-lasting activation of neuronal populations; it is accompanied by changes in the level of transcription of the late genes, formation of plastic modifications in the brain, and stabilization of motor skills [16]. It was shown earlier that motor learning results in plastic changes in groups of GABA-ergic neurons and in increase in the density of inhibitory terminals on the somata and dendrites of motoneurons in experimental animals [8, 17]. Our study indicated that long-lasting (12 days) trainings with repetitive realization of food-procuring operant forelimb movements induce in such animals considerable changes in the level of *c-fos* expression in the dorsal and ventral horns of the cervical enlargement of the spinal cord. Relatively high densities of Fos-ir neurons were observed in the *substantia gelatinosa* (layer 2i) and *nucl. proprius* (laminae 3 and 4), and also in layers 6 and 9 (Fig. 1 B). Such type of the laminar distribution of Fos-ir neurons is significantly different from the patterns observed after nociceptive and fatiguing muscle stimulation; in the latter cases, the maxima of Fos-immunoreactivity are localized within

superficial layers of the gray matter (1 and 2o) and lateral regions of the neck of the dorsal horn (lamina 5). Under conditions of nociceptive stimulation of the limb muscles, Fos-immunoreactivity was not detected in motoneurons [7, 18, 19]. As was shown earlier, long-lasting walking of rats on a treadmill (with no significant muscle fatigue) results in activation of neurons in layers 2i, 3–5 (medial part), and 7 and 9 of the gray matter [2]. Neurons of these layers play a crucial role in the control of locomotion and realization of a few other motor programs [4, 5, 20].

In our study, Fos-immunoreactivity was also detected in the LSp zone. In rodents, this nucleus is localized near the dorsal part of the lateral horn; it is mostly involved in transmission of nociceptive information to the thalamus; neurons of this nucleus are also sources of direct projections to the hypothalamus. Therefore, the LSp and zone around the *canalis centralis* (lamina 10) function as integrative centers of autonomic regulation providing homeostatic effects. Neurons of these regions influence the development of motivational/affective phenomena directly related to autonomic reactions [4, 15, 21, 22]. Recently, we found that the performance of operant food-procuring movements is accompanied by phasic and tonic cardiovascular responses with the presence of a stable parasympathetic component within the final phase of such movements (when capturing food globules) [21].

Thus, we observed significant intensification of Fos-immunoreactivity in the cervical spinal segments of rats realizing repetitive food-procuring movements after preliminary systemic introduction of 7-NI. It can be hypothesized that such intensification is related to disinhibition of segmental interneurons activated in the course of operant movements and with increase in the responsiveness of interneurons with respect to muscle proprioceptive and descending supraspinal impulsation. It should be emphasized that no intensification of *c-fos* expression after injections of 7-NI was observed in animals that did not perform the above motor activity. It should also be noted that NO is produced, in particular, by brainstem neurons localized in proximity to cell sources of serotonin-, noradrenaline-, and GABA-ergic projections to the spinal cord [23]. Thus, some effects of increase in *c-fos* expression in the spinal cord under conditions of blocking of nNOS are, probably, determined by metamodulatory influences on spinal neuronal networks from the descending aminergic and GABA-ergic systems of the brainstem.

All experimental procedures were carried out in agreement with the European Directive of the Council of Communities (November 24, 1986; 86/609 ECC) and statements of the Committees on bioethics in the Bogomolets Institute of Physiology (NAS of Ukraine), Pirogov Vinnitsa National University (Ministry of Public Health of Ukraine), and Mechnikov Odessa National University.

The authors of this paper, A. V. Maznichenko, O. V. Vlasenko, Ye. P. Man'kovskaya, T. V. Buzyka, and V. A. Maisky, confirm that they have no conflict of interest.

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