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NADPH-diaphorase reactivity and Fos-immunoreactivity within the ventral horn of the lumbar spinal cord of cats submitted to acute muscle inflammation induced by injection of carrageenan

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ABSTRACT

The NADPH-diaphorase activity and Fos-immunoreactivity within the ventral horn of the lumbar spinal cord were studied in cats with acute unilateral myositis following injection of carrageenan into the *m.m. gastrocnemius-soleus*. In carrageenan-injected cats maximum in the mean number of intensely stained NADPH-diaphorase reactive (NADPH-dr) neurons was found in lamina VII (+100%) and VIII (+33%) of the contralateral ventral horn of the L6/L7 segments as compared with control animals. The maximumal level of Fos-immunoreactivity was registered in the same laminae with ipsilateral predominance (39.3 ± 4.6 and 7.6 ± 0.9 cells), in comparison with the contralateral side (13.6 ± 0.8 and 5.5 ± 0.6 cells, respectively; $P < 0.05$). We also visualized low-intensely stained and double labelled (Fos immunoreactive + low-intensely stained NADPH-dr) multipolar and fusiform Renshaw-like cells (RLCs) within the ventral horn on both sides of the L6/L7 segments in carrageenan-injected cats. We visualized the double labelled (Fos-ir + NADPH-dr) multipolar and fusiform Renshaw-like cells (RLCs) within the ventral horn on both sides of the L6/L7 segments in carrageenan-injected cats. A significant difference in the mean number of RLCs was recorded between the ipsi- and contralateral sides in the lamina VII (13.6 ± 2.5 *vs.* 4.9 ± 0.7 cells, respectively). We suppose that activation of inhibitory RLCs in ipsilateral lamina VII could be directed on attenuation of activation of motoneurons during muscle pain development. Our study showed that a significant contralateral increase in the number of NADPH-dr cells is accompanied by an ipsilateral increase in c-Fos expression in lamina VII. These data may suggest that NADPH-dr neurons of the contralateral ventral horn through commissural connections also involved in the maintenance of the neuronal activity associated with acute muscle inflammation. It is also hypothesized, that during acute myositis, plastic changes in the ventral horn activate the processes of disinhibition due to an increase in the number of NADPH-d-reactive neurons in the spinal gray matter.

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1. Introduction

Signals from muscle afferents of Ia and II groups are the important peripheral messengers from the muscles to the CNS, which initiate generation of intrasegmental and supraspinal commands directed toward steering of posture and gait (Proske et al., 2000). The pain signals from muscle afferents of III and IV groups contribute to the formation of specific intersegmental neuronal activation and changes of c-Fos expression and NADPH-diaphorase reactivity in different laminae of the spinal cord. In case of muscle inflammation, the pain hypersensitivity is depended on sensitization of primary afferent fibers and hypersensitivity of nociceptive neurons, which are controlled, by brain stem and spinal cord centers (Kidd and Urban, 2001). In our previous studies (Schomburg et al., 2007, 2015) in the case of acute carrageenan-induced

Abbreviations: NADPH-dr, NADPH-diaphorase reactive; Fos-ir, FOS-immunoreactive; RLCs, Renshaw-like cells; GS, *m.m. gastrocnemius-soleus*; DAB, 3,3′-diaminobenzidine tetrahydrochloride.

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inflammation of the *m.m. gastrocnemius-soleus* (GS) in cats, it has been found a significant increase in the amplitude (>100%) of the monosynaptic reflexes of flexors and extensors. These reflex changes could be mirrored in the enhanced endothelial and neuronal activation within the ventral horn of the spinal cord (Cirino et al., 2003; Chacur et al., 2009).

It is known that the NADPH-diaphorase activity reflects, to some extent, the activity of nitric oxide (NO) synthase in the spinal cord of rats and cats (Vincent and Kimura, 1992; Pun et al., 1993). The production of NO in the spinal cord and increase in expression of NOS 1, regulated by NOS 2, could be responsible for the maintaining of persistent inflammatory and neuropathic pain (Freire et al., 2009; Hervera et al., 2010; Schmidtko, 2015). NADPH-diaphorase exists in distinct subsets of neurons in feline brain demonstrate the different intensity of histochemical staining (Mizukawa et al., 1989). In the cervical and lumbar spinal cord of agouti were defined two different types of NADPH-diaphorase reactive neurons – with large, heavily stained cell bodies (type I) and relatively small and poorly stained somata (type II). The intensely stained neurons located mainly in the dorsal horn and around the central canal whereas in the ventral horn were found a few such cells (Freire et al., 2008). In pheasant thoracic spinal cord within the ventral horn also possessed a number of scattered, intensively stained neurons (Kluchová et al., 2001). In cats with chronic muscle inflammation, we have earlier recorded a marked decrease in NADPH-diaphorase activity within superficial laminae of the dorsal horn on the ipsilateral side of the L6/L7 segments (Steffens et al., 2007). However, the population of spinal NADPH-diaphorase reactive (NADPH-dr) neurons in the case of muscle pain are capable of providing a multidirectional response – an increase or a decrease in their number in the gray matter of the spinal cord (Hoheisel et al., 1997; Pilyavskii et al., 2005; Steffens et al., 2007). The main interest of many researches in case of pain development was focused on changes in the distribution patterns and mean number of NADPH-dr cells within the dorsal horn and area around central canal, which receive inputs from visceral and cutaneous noxious afferents (Freire et al., 2009). However, the data about changes in the level of NADPH-diaphorase activity within the ventral horn of feline spinal cord during acute muscle inflammation are not sufficient. Thus, the aim of the present study was to reveal the laminar distribution of intense and low-intense stained NADPH-dr neurons and Fos-immunorective cells (as a marker of neuronal activation) within the ventral horn of the lumbar segments in cats with acute unilateral carrageenan-induced myositis.

2. Materials and methods

2.1. Subjects

The use of the animals was approved by the Ethics Committee of the Institute and performed in accordance with the European Communities Council Directive, November 24, 1986 (86/609/EEC). Experiments were performed on nine male cats (*Felis domesticus*) weighing 2.4–4.5 kg, with ages ranging from 1 to 2 years. The animals were purchased from a state-controlled animal farm through the common animal facility of Bogomoletz Institute of Physiology (Kyiv). Animals were divided into three groups: (1) control group of intact cats (n = 3); (2) sham-operated group (n = 3); and (3) carrageenan-injected group (n = 3). In order to prevent the influences of descending signals from supraspinal sources on the lumbo-sacral spinal cord in animals of groups (2) and (3) all procedures were performed in the high-spinalized cats (Schomburg and Steffens, 2002).

2.2. Procedure

Anesthetized (ether–halothane) animals of sham-operated and carrageenan-injected groups were tracheotomized, paralyzed with pancuronium (Organon, USA, 0.1–0.2 mg/kg, i.v.), artificially ventilated and high-spinalized at C1. Mean arterial blood pressure and body temperature were continuously monitored and kept at physiological levels about 80 mm Hg and 37–38 °C, respectively. The spinalized cats were then allowed to stabilize the neuronal activity and basal level of c-Fos expression in the lumbar cord for 8 h after laminectomy. The acute muscle inflammation was induced by infiltrating the left GS muscle with a 2% carrageenan (kappa carrageenan, Sigma-Aldrich, St. Louis, MO, USA) suspended in Ringer's solution. The carrageenan solution was injected into both heads of the GS muscle (three injections of 0.5 ml into each head) (Schomburg et al., 2007, 2015).

The control, sham-operated (subjected to standard surgery, but without carrageenan injection) and carrageenan-injected cats (4 h after carrageenan injection (Schomburg et al., 2007)) were deeply anesthetized with sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA; 90 mg/kg, i.p.) and perfused through the ascending aorta, first with phosphate buffer saline (500 ml, which contained 0.2% sodium nitrite and 25,000 u/l of heparin) followed by fixative solution (1500 ml) containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Segments of the lumbar spinal cord (L6 and L7) were quickly removed, additionally postfixed for 12 h in the same fixative, and cryoprotected at 4° C for 48 h in 30% sucrose solution. Frontal frozen 40 μm-thick sections of the L6 and L7 were cut using a freezing microtome. About 50–60 serial sections from each lumbar segment were immunohistochemically (c-Fos) and histochemically (NADPH-diaphorase) stained.

2.3. Immunohistochemical/Histochemical procedures

Fos-ir nuclei of neurons were detected in spinal sections according to a standard avidin-biotin-peroxidase technique using a primary rabbit polyclonal antibody against c-Fos protein (1:2000, Ab-5, lot# 4191-1-1, Oncogene Research Products, San Diego, CA 92121, USA) and secondary antibody (1:200, biotinylated anti-rabbit IgG made in goat, ABC Kit, PK 4001, Vector Laboratories, Burlingame, CA, USA) (Hsu et al., 1981). Fos-ir nuclei were visualized with nickel-intensified 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) and detected as black nuclei. Labeled neurons were counted under a light microscope; their laminar distribution was established using the schemes of feline spinal cord (Brown, 1981). To evaluate the possibility of double-labelling of Fos immunoreactivity and NADPH-diaphorase reactivity in neurons, every second immunostained section of the L6 and L7 was additionally incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.3% Triton X-100, 0.2 mg/ml nitroblue tetrazolium (Sigma-Aldrich, USA), and 0.5 mg/ml β-NADPH tetra-sodium salt (Sigma-Aldrich, USA) at 37° C for 30–60 min (Vincent and Kimura, 1992). NADPH-dr neurons within sections of the lumbar spinal cord were detected under light microscope by blue color of their cytoplasm and unstained nuclei.

2.4. Statistical analysis

Up to 25–30 double-stained (Fos-ir + NADPH-dr) sections from the L6/L7 per animal of each group were analyzed. The mean number ± SEM of labelled cells per section was calculated in laminae VII–X of the gray matter on the ipsi- and contralateral sides of the ventral horn in the L6/L7 segments obtained from cats of different groups. The possible errors due to double-count of the same cell in neighboring section were corrected using the Abercrombie

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