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EXPERIMENTAL ARTICLES

Neurochemical Changes in the Rostral Ventromedial Nucleus of the Medulla Oblongata in Rats with Developing Neuropathic Pain

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Abstract—The dynamics of neurochemical rearrangements in neurons in the ventromedial reticular formation of the medulla oblongata in rats during the development of neuropathic pain were investigated. Distinct populations of monoaminergic and NOergic projecting neurons and cells that metabolize agmatine were found in the nucleus. The change in the activity of these neurons during the initiation (week 1) and formation (4 weeks) of neuropathic pain has several phases. Decreased synthesis of catecholamines, increased catabolism of agmatine, and activation of NO synthesis were found in neurons by the end of the first week. At 4 weeks after the surgery, the parameters of the catecholaminergic system returned to the control level, NOergic activity was significantly reduced and the catabolism of agmatine continued to increase. These findings suggest the involvement of bulbar NOergic, mono- and polyaminergic neurotransmitter mechanisms in the initiation and maintenance of neuropathic pain.

Keywords: NO-synthase, catecholamines, agmatine, ventromedial reticular formation, neuropathic pain
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INTRODUCTION

The nuclei of the reticular formation play a strategically important role in the integrative systemic response to pain by transferring signals from the mesencephalon and the diencephalon to neurons in the posterior horns of the spinal cord and thus regulate the activity of the "spinal gate of pain" [1–4]. The molecular, morphological, and physiological basis of the endogenous antinociceptive system is actively studied. Its main effector component with direct projections to local and projecting neurons of the posterior horn is the complex of rostral ventromedial nuclei of the medulla oblongata (RVMN), which is located in the region of the nucleus raphe magnus and the adjacent paragigantocellular reticular formation [5]. The work of this center modulates the intensity of ascending nociceptive signals, regulates pathological pain phenomena, including thermal and tactile allodynia, provides the transformation of tonic pain to its chronic type, and underlies the formation of the paradoxical effects of opioid analgesics, viz., the development of pro-nociceptive activity. Such a wide range of biological effects of this nucleus depends on the specific organization of its local afferent projections, which provides the functioning of two opposite mechanisms: endogenous analgesia and endogenous pro-nociception [6, 7]. Molecular, neurotransmitter, and receptor changes that occur in this nucleus transform pain into a dynamic process that regulates the intensity of the pain and its sensitivity to treatment. Many adjuvant analgesics (anticonvulsants, reuptake inhibitors, and gabapentin and its analogs) are considered to mimic the activity of this particular morphological and functional system to some extent [7]. Despite the great success in revealing the electrophysiological properties of RVMN neurons, the molecular and neurotransmitter bases of endogenous bulbar modulation of pain have still not been completely investigated.

The purpose of this study was to investigate the dynamics of neurochemical rearrangements in neurons of the rostral ventromedial nucleus of the medulla oblongata during the development of neuropathic pain.

MATERIALS AND METHODS

The characteristics of the experimental subjects. Our study was performed with male rats that weighed 250 g; the experiments were approved by the bioethics committee of the Institute of Marine Biology. The study was performed in accordance with the rules on the use of experimental animals (Annex to the Order of the Ministry of Health of the USSR no. 755 from August 12, 1977). The animals were kept in a vivarium in accordance with the "Sanitary rules on the organi-

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zation, equipment, and maintenance of experimental biological clinics" (from April 6, 1993).

An experimental model of chronic pain (the application of three tight silk ligatures on the sciatic nerve of the right hind paw of the rat) was used in the study. It induces a group of neuronal events, whose dynamics are similar to neuropathic pain in humans [8]. The development of pain in animals is accompanied by a number of pathological symptoms, which can be quantitatively measured and serve for the evaluation of pain intensity [9]. Previously, we described the dynamics of the main manifestations of pain in experimental animals in the cold-plate test and the disability test (cold allodynia, spontaneous pain, and motor deficit) [10]. In this study, the evaluation of neurochemical changes in the brain was performed at time periods that correspond to the initiation of neuropathic pain (1 week after surgery) and its development (4 weeks).

Immunohistochemical. The animals were divided into two groups ($n = 21$): the control group ($n = 7$) included intact animals and the pain group ($n = 14$) included animals with neuropathic pain. Animals were anesthetized by intraperitoneal injection of sodium thiopental (3%, 60 mg/kg) and then perfused with 4% paraformaldehyde prepared with 0.1 M phosphate buffer (pH 7.2). To evaluate the neurotransmitter profile of RVMN neurons, immunoperoxidase staining was used to identify tyrosine hydroxylase (TH), the neuronal form of NO-synthase (nNOS), and agmatinase, an enzyme of agmatine metabolism (AGM). The immunohistochemical protocol included the following steps: fixation of tissue samples (4% paraformaldehyde prepared with 0.1 M phosphate buffer, pH 7.2) for 12 hours and a subsequent wash; serial cryostat sectioning to 50 μm-thick slices; pre-incubation in solutions that block endogenous peroxidase activity and nonspecific antibody binding; processing of the slices in a primary antibody solution (4°C, 24 hours); and incubation with the secondary antibody and the immunoperoxidase reaction. We used primary polyclonal mouse and rabbit antibodies against AGM (Sigma-Aldrich, SAB1102156, United States), TH (Vector Labs, T 489, United States) and nNOS (Abcam, ab40662, United States) diluted to 1 : 500, 1 : 30, and 1 : 2000 respectively. Secondary antibodies labeled with horseradish peroxidase (Vector Laboratories, PI-1000 (anti-rabbit); PI-2000 (anti-mouse), 1 : 300) were used according to the manufacturer's instructions. For carrying out the immunoperoxidase reaction, the chromogen (Thermo scientific, TL-060-QHD, United States) was used. After staining, sections were thoroughly washed with 0.1 M phosphate buffer (pH 7.2), dehydrated, and embedded in balsam according to the standard protocol.

Morphological. Calculation of the total number of neurons in the RVMN was performed with Nissl-stained slices. Slices were stained with gallocyanin-chrome alum (a 0.15% solution of gallocyanin in a 5% solution of chrome alum); the sections were then dehydrated in alcohol and embedded in balm following the standard protocol. Mounted slices were viewed using an AxioScope A1 light microscope (Carl Zeiss, Germany) and photographed with an AxioCam ICc3 digital camera (Carl Zeiss, Germany).

Quantitative data analysis. The number of neurons in the rostral ventromedial medulla nucleus was counted in every fifth serial section using a 12.5× objective, which allows one to obtain images of the entire nucleus. We counted the absolute number of immunopositive cells and calculated the percentage they took of the area of the nucleus (the specific density of neurons in 1 mm^3) and the number of Nissl-stained neurons (% content). Only cells with a nucleus were counted. The morphometric analysis was performed using ImageJ 1.41 software. The statistical analysis was performed using one-way ANOVA (Tukey post-hoc test) in GraphPad Prism 4.00 software. Results were considered significant at $p < 0.05$.

RESULTS

Neuronal NO-synthase. The immunohistochemical staining of NO-synthase in the rostral ventromedial nucleus revealed labeling of large (30 μm or more) and medium-sized (15–30 μm) neurons; their number was 16.9 ± 0.5% of the number of Nissl-stained neurons. Small interneurons (15 μm) were not stained with NO-synthase-specific antibodies (Fig. 1a). Histochemical localization of NO-synthase clearly distinguished RVMN neurons from the neurons of the other adjacent segments of the reticular formation, where this enzyme was not detected. On the 7th day after the application of the ligature on the sciatic nerve in the "pain" group, the number of nNOS-positive neurons in the rostral ventromedial nucleus increased by 26% in comparison with the control group (451.7 ± 29.9 neurons per 1 mm^3) (Figs. 1a, 1b, and 2). By the 28th day the number of nNOS-positive neurons decreased by 20% compared to the intact animals (Figs. 1a, 1b, and 2).

Tyrosine hydroxylase. A fairly large population of TH-positive neurons was immunostained in the ventral reticular formation (17.5 ± 1.5% of the number of Nissl-stained neurons). They belonged to the group of medium and large multipolar neurons, which were mostly found in the ventromedial part of the nucleus (Fig. 1d). The population of TH-positive neurons underwent significant changes in the neuropathic pain development. On the 7th day after ligation of the sciatic nerve, the number of TH-positive neurons in the RVMN of animals from the pain group decreased by 19% compared to the control animals (436 ± 30.8 neurons per 1 mm^3) (Figs. 1d, 1e, and 2). This parameter returned to the intact control level by the 28th day (Figs. 1d, 1e, and 2).

Agmatinase. In the RVMN, agmatinase stained only the large neurons (6.7 ± 0.6% of the Nissl-stained

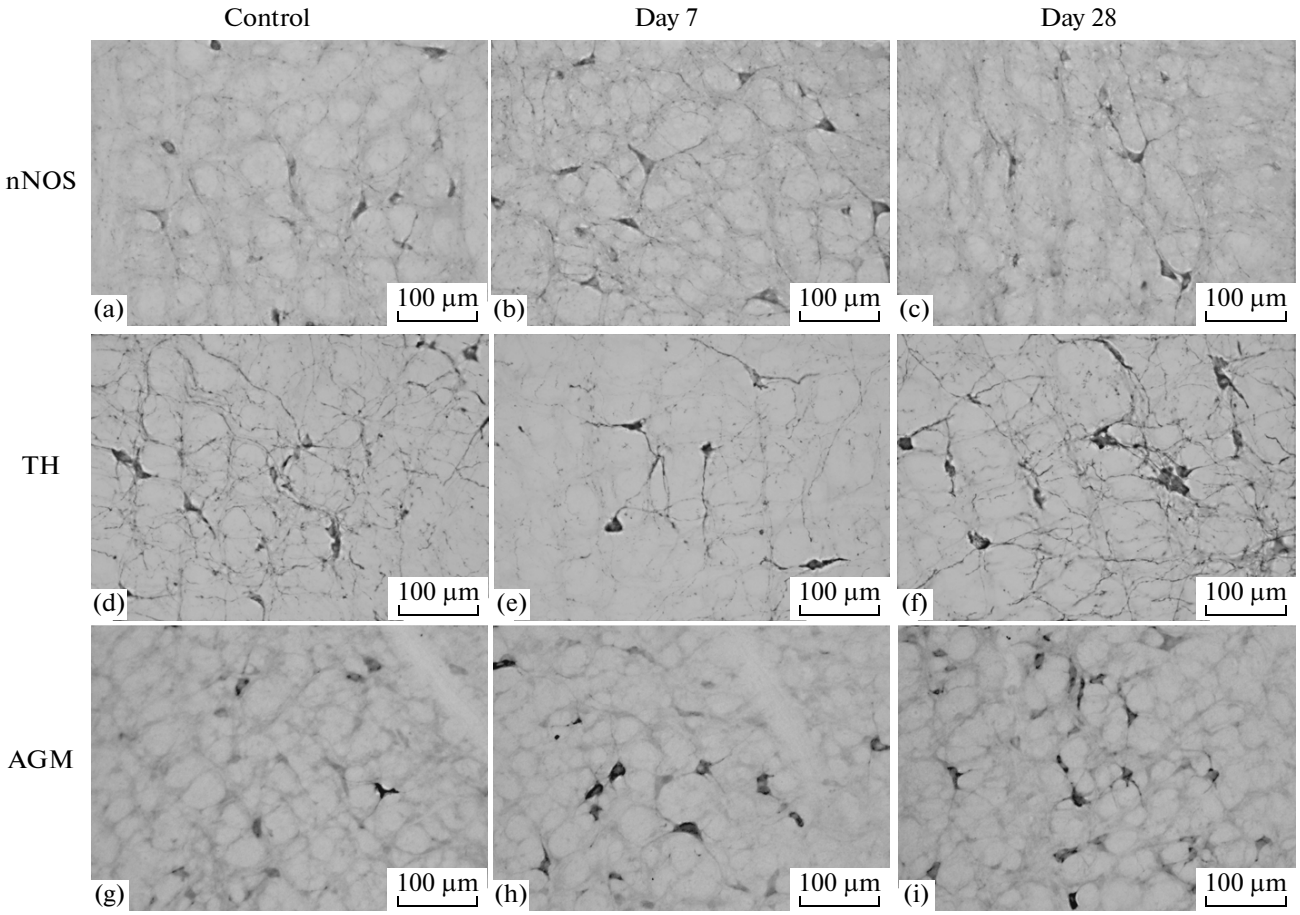

Fig. 1. nNOS, TH, and AGM-positive neurons in the rostral ventromedial nucleus of the medulla oblongata in normal rats (a, d, g) and in the development of neuropathic pain on day 7 (b, e, h) and day 28 (c, f, i) after ligation of the sciatic nerve.

neurons). The number of agmatinase-positive neurons increased in the rostral ventromedial nucleus at all experimental time points after the damage to the sciatic nerve. On the 7th day after application of the ligature on the sciatic nerve, the number of agmatinase-positive neurons in the RVMN of the "pain" group increased by 42% in comparison with the intact animals (430.5 ± 43.1 neurons per 1 mm^3) (Figs. 1g, 1h, and 2). This parameter increased by 87% in comparison with the control group by the 28th day after ligation (Figs. 1g, 1i, and 2).

DISCUSSION

The presently known data on the functional heterogeneity of RVMN neurons can, to some extent, explain their ambiguous role in the modulation of the pain process. According to the hypothesis of GABAergic release [6], the effectiveness of endogenous analgesia depends on the functioning of GABAergic neuron circuits in the central gray matter of the midbrain and rostral ventromedial nucleus. In the RVMN region, so-called cell switches, or ON-cells and OFF-cells, and neutral cells have been identified. They perform

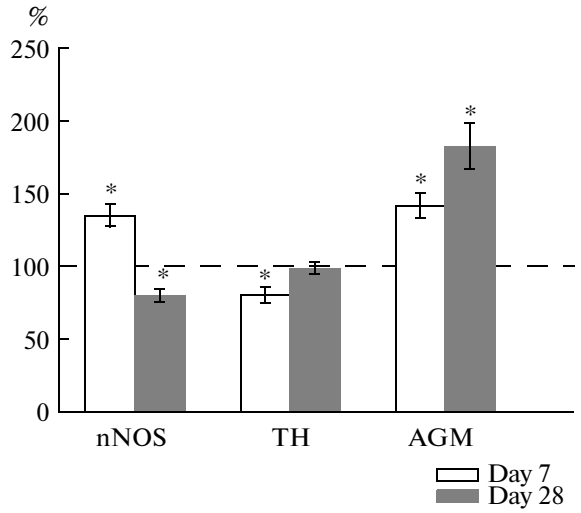

Fig. 2. Changes in the activities of nNOS, TH, and AGM-positive neurons in the rostral ventromedial nucleus in animals with neuropathic pain (the control group is shown with a dotted line, the specific density of the distribution of nNOS, TH, and AGM-positive neurons is taken as 100%). * Significant differences as compared to control animals ($p < 0.05$).

pro- or antinociceptive modulation of the activity of the "spinal gate of pain" [11]. GABAergic cells are considered to be ON-cells, while OFF-cells include neurons that send projections to the spinal cord and modulate the activity of its nociceptive mechanisms. The problem of the determination of their chemical phenotype and dynamic rearrangements under the effect of pain requires a detailed investigation. It is possible that the resulting modulating effect of projecting neurons of this nucleus (anti- or pro-nociceptive) is determined by the intensity and origin of afferentation and also by the local balance of neurotransmitters (or other neuroactive substances) at different time points during the pathological pain process.

In this study, the dynamics of the activities of the three neurotransmitter systems in the neurons of the rostral ventromedial medulla nucleus (monoaminergic, NOergic, and agmatinergic) were investigated at the initiation of neuropathic pain and at the peak of its development. Each of the investigated mediators can be regarded as an important participant of the integrative pain response. NOergic signaling in the nerve centers at the spinal level and at the level of centripetal signal transmission leads to the intensification, multiplication, and prolongation of the pain. Activation of NO-synthase in spinal pain centers positively correlates with the intensity of the pain [12]. However, the effect of NO in supraspinal centers may be the opposite and activation of the enzyme occurs due to an increase in the activity of endogenous analgesic systems [13, 14]. Descending catecholaminergic projections in the spinal cord are direct modulators of the pain gates and are involved in stress-induced analgesia. On the other hand, the lack of endogenous noradrenaline contributes to the chronic-pain process [7]; and associated sensitization and expression of adrenergic receptors in the primary sensory fibers can enhance the effect of noradrenergic analgesia and underlie the development of the pathological phenomena of pain, viz., hyperalgesia and allodynia [15]. The biogenic amine agmatine is a recently discovered brain neurotransmitter. It is considered to play an important role in endogenous analgesia, especially in neuropathic pain [16–29]. Recent studies of the mechanisms of its antinociceptive action suggest that this phenomenon depends on the direct influence of the amine on NO metabolism, the activity of imidazoline, and NMDA-glutamate receptors [20–22].

Our immunohistochemical study discovered populations of TN-, NOS-, and agmatinase-positive neurons in the rostral ventromedial nucleus. Our preliminary results on the co-localization of the three markers in the RVMN indicate that the used antibodies detected distinct populations of neurons. Their size, localization, and morphology put them in the category of projecting cells. The exact regions of their projections in the spinal cord have yet to be discovered, but the contacts between the axons of RVMN neurons and local and projecting neurons in the posterior horns of the spinal cord, as well as the spinal terminals of primary sensory nerve fibers, have been described [23].

Changes in the activity of these neurotransmitter systems of the RVMN during initiation (1 week) and development (4 weeks) of the pain syndrome include several phases. On the 7th day (at the peak of pain activity) activation of nNOS- and agmatinase-positive cell populations of RVMN occurs simultaneously with a decrease in the activity of tyrosine hydroxylase. At 4 weeks after the surgery (when pathological cold allodynia resolves completely, but the asymmetry in the maintenance of the body weight persists, and tactile allodynia is activated), the parameters of the catecholaminergic system return to the level of the intact animals, NOergic activity significantly declines, and agmatine catabolism continues to grow. These results show that nitric oxide, catecholamines, and agmatine are involved in the descending modulation of a pain signal, along with the well-known GABA, glutamate, neurokinin, opioid, and serotonergic mechanisms of bulbar pain control that have been characterized in detail [11, 24]. The dynamics of the activity of these neurotransmitters under the conditions of neuropathic pain is different from the dynamics of the development of nociceptive and acute inflammatory pain, in which a decrease in the activity of NO-synthase, an increase in noradrenaline metabolism [25], and an increase in the local concentration of agmatine (unpublished observation) are predominant. Our work has shown that the activity of catecholamine synthesis significantly decreases and NOergic transmission (especially in the initial period) increases in the chronic-pain process. Such a neurotransmitter profile may indicate the exhaustion of endogenous analgesic resources (or activation of pro-bulbar pain mechanisms) and cause an increase in pain intensity. By the 4th week of observation, neurotransmitter conditions for the resumption of endogenous analgesic protective mechanisms are formed due to a drastic decrease in the NOergic activity of the nucleus and restoration of the intact catecholamine levels. Certain pathological pain symptoms (cold allodynia) completely disappear in animals in this period [10].

The synchronous and multidirectional dynamics of the enzyme activity of nitric oxide and catecholamine synthesis that were found in our study suggests that reciprocal modulating effects can occur between these neurotransmitter systems at the level of the nucleus. Complex and multidirectional metabolic and synaptic interactions have been described between the NO and noradrenergic neurotransmission systems: nitric oxide is able to activate the basal release of noradrenalin, but it decreases its stimulated synthesis in neurons [26] and reduces the production of catecholamines in the adrenal chromaffin tissue [27]. NO interaction with noradrenaline is also ambiguous at the level of adrenergic receptors. The synaptically inactive metabolite 6-nitro-noradrenaline is rapidly converted to noradrenaline under the influence of NO, which, in turn,

inhibits neurotransmitter reuptake and prolongs its action in the synaptic cleft [28]. We assume that prolonged neuropathic pain alters the activity of intracellular signaling systems in neurons of the reticular formation and provides the development of reciprocal inhibitory interactions in the NO-monoamines system.

Unlike NO- and monoaminergic neurons that exhibit phase dynamics of activity during the development of neuropathic pain, the system of agmatine metabolism activity in RVMN neurons steadily increases. Despite the considerable number of studies that have investigated the analgesic effect of exogenously administered agmatine sulfate [16, 17], the data on the localization, synaptic and metabolic interactions, and the dynamics of the activity of agmatinergic neurons in the brain are scarce [29]. Agmatinase, whose activity in RVMN neurons increases at all stages of the development of neuropathic pain, is an enzyme that degrades the endogenous analgesic and antidepressant agmatine. To interpret the results of our work, more detailed studies on the localization, morphology, and neuronal interactions of agmatinergic RVMN neurons are obviously required. However, we believe that in neuropathic pain the increased agmatine degradation that has been observed in the neurons of the important bulbar center of endogenous pain modulation is direct evidence of a decrease in its analgesic activity.

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