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Changes in the Gene *c-fos* Expression in the Rat Spinal Cord after Suppression of Activity of the Cerebral Monoaminergic Systems

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We detected changes in the gene *c-fos* expression induced by activation of muscle afferents in the rat spinal cord after systemic introduction of reserpine (irreversible suppressor of vesicular transporter of monoamines, 1.5 mg/kg, intraperitoneally) and with no action of this agent. Numbers of Fos-immunoreactive (Fos-ir) neurons were calculated in the gray matter of the lumbar spinal segments after unilateral vibrational stimulation (VS) of the Achilles tendon of the *mm. gastrocnemius–soleus*. In the spinal cord of rats preliminarily injected with reserpine, greater numbers of Fos-ir neurons were observed; these units were localized mostly in layers 4–7 and in the *nucl. intermediolateralis* (35.4 ± 1.6 and 16.7 ± 0.9 positive neurons per 40 μm-thick slice) of segments L1–L2, and also in layers 4–7 and layer 9 (Fos-ir motoneurons) of segments L4–L5 (51.7 ± 3.4 and 11.4 ± 1.5 labeled units, respectively). The numbers of activated cells in the above structures of the spinal cord after VS but without preliminary injections of reserpine were, on average, 25.6 ± 1.4 and 3.5 ± 0.5, 27.8 ± ± 0.9 and 6.9 ± 0.3 units, respectively. Most ipsilateral Fos-ir motoneurons (86%) were localized in the lateral pool of layer 9, and only 14% of labeled motoneurons were localized in its medial regions. The results obtained show that weakening of monoaminergic influences resulting from administration of reserpine is accompanied by increase in the activity of intraspinal neuronal networks activated by proprioceptive afferent volleys, and the effects of the above inputs on spinal motoneurons and sympathetic preganglionic neurons are intensified. Weakening of inhibitory control, realized by inhibitory interneurons in the pathways of transmission of excitatory influences from muscle afferents to motoneurons, which was observed in our experiments after suppression of monoaminergic modulatory systems, can be considered a significant factor responsible for the development of rigidity/spasticity of the limb muscles manifested in neurodegenerative diseases and after traumas of the spinal cord.

Keywords: expression of gene *c-fos*, lumbar spinal cord, vibrational stimulation, reserpine, suppression of the central monoaminergic modulatory system.

INTRODUCTION

Descending influences from the cerebral monoaminergic systems represent an important component of descending control of the activity of spinal locomotor (and motor in general) centers; adequate locomotion is realized only under conditions of coordinated release of dopamine, noradrenaline, and serotonin in different regions of the spinal cord [1-3]. Effects of different monoamines are mediated via specific receptors; each class of monoaminergic receptors modulates a relatively extensive and, at the same time, specific range of kinematic, kinetic, and myographic characteristics of the movement [4-6]. It is important that the action of some monoamines can accelerate recovery of the disturbed locomotor function after trauma to the spinal cord [7-9]. Effects of certain monoamines at a segmental level are accompanied by significant changes in the activity of spinal neurons involved in transmission of information from segmental proprioceptors [10-14].

As is known, elimination or modulation of the influences from one descending pathway or another results in changes in the balance between excitatory and inhibitory influences of peripheral afferents in spinal neuronal networks and in disorders

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of interaction between various muscle groups; this is manifested in disturbances of posture and movements [5]. Weakening of the functions of the dopaminergic system is one of the main reasons for the development of locomotion disorders manifested in Parkinson's disease [15].

Proprioceptors of the skeletal muscles, in particular receptors of intrafusal spindles, are important sources of signals to the segmental systems of motor control. Such signals initiate generation of intraspinal and suprasegmental commands directed toward postural and locomotion regulation [16-18]. Shifts in the activity of spinal neuronal populations induced by long-lasting/intense stimulation of segmental peripheral afferents are reflected in intensification of the expression of "early" genes, gene *c-fos* first of all. These shifts can be noticeably modified under the influences of modulatory neurochemical systems. In particular, it was shown recently that, after systemic application of 7-nitroindazole (a blocker of neuronal NO-synthase), intensified expression of protein c-Fos induced by influences from low-threshold muscle afferents is additionally increased [19]. After treatment with reserpine, activation of nociceptive neurons in the spinal cord increases, and the level of c-Fos expression in suprasegmental catecholaminergic and non-catecholaminergic neurons localized in different brain regions also increases [20, 21]. At the same time, the question of how populations of central neurons involved in transmission of information from muscle spindle receptors in segmental neuronal networks react to changes in the intensity of effects from the monoaminergic cerebral systems remains obscure from certain aspects.

As was found, application of reserpine, an irreversible suppressor of vesicular monoamine transporter, results in rapid release of serotonin, noradrenaline, and dopamine from presynaptic terminals in the loci of projections of aminergic fibers and in subsequent suppression of synthesis of these monoamines in neurons of the corresponding cerebral monoaminergic systems [22, 23]. This finally results in the development of the symptoms of fibromyalgia, rigidity, tremor, and akinesia. Manifestations of such symptoms are typical of Parkinson's disease in humans and analogous pathological shifts under conditions of modeling of this disease in animals [15, 24-26]. As was assumed, the development of reserpine-induced disorders of motor functions can be related not only to changes in the supraspinal structures but also to reserpine-related disregulation of motor control processes at a segmental level.

Trying to elucidate possible modifications in the functioning of segmental networks controlling the motor activity after weakening of the function of monoaminergic systems, we carried out a comparative analysis of the patterns of distribution of Fos-ir neurons in the lumbar part of the rat spinal cord upon activation of receptors of the hindlimb muscles. Such activation was provided by long-lasting vibrational stimulation (VS) of the tendon of the *triceps surae* (*mm. gastrocnemius-soleus*, GS). The above patterns were examined in animals after isolated action of VS and in the case where such stimulation was preceded by systemic introduction of reserpine.

METHODS

Immunohistochemical detection of activated neurons within lumbar segments of the spinal cord was carried out in five groups of male Wistar rats. Two groups served as control. Intact animals ($n = 4$) were included in the first group; rats of group 2 ($n = 4$) were anesthetized by chloral hydrate (Sigma, USA; 400 mg/kg, i.p.) and injected (also i.p.) with 1.0 ml of 0.9% NaCl solution. Animals of the third group ($n = 4$) were also anesthetized with 400 mg/kg chloral hydrate and subjected to long-lasting VS for activation of spinal neurons. The separated Achilles tendon of the *triceps surae* (*mm. gastrocnemius-soleus*, GS) was vibrated for 30 min; sinusoidal vibration was used. One-min-long periods of vibration were alternated with one-min-long intervals of resting. The frequency and amplitude of VS were 100 sec^{-1} and 0.5 mm, respectively. In animals of group 4 ($n = 4$), the laminar distribution of activated (Fos-ir) neurons was examined 4 h after double injections of reserpine (1.5 mg/kg, i.p.). Animals of the fifth group ($n = 4$) were anesthetized by chloral hydrate 1 h after injections of reserpine; then, VS was applied to the GS tendon. After the above-described manipulations, animals of group 2, 3, and 5 were in the resting state during 2 h. As is believed, such time interval is sufficient for synthesis and accumulation of Fos protein in the activated neurons.

After the above experimental procedures, animals

of all groups were perfused intracardially (via the ascending aorta) under deep anesthesia (sodium pentobarbital, Sigma, USA; 90 mg/kg i.p.). For perfusion, a phosphate saline buffer (PSB) with the addition of 0.2% sodium nitrite and 25,000 U/l heparin was first used. Then, perfusion was continued with 4% paraformaldehyde in 0.1 M PSB (pH 7.3). Segments of the lumbar enlargement (L1–L5) of the spinal cord of each animal were rapidly excised and additionally fixed during 12 h; then, for cryoprotection, slices were immersed for 48 h in cold (4°C) sucrose solution in 0.1 M PSB. Forty-μm-thick slices were prepared using a freezing microtome and collected in wells with 0.1 M PSB for subsequent immunohistochemical staining.

Fos-ir nuclei of the activated neurons were identified using an avidin-biotin-peroxidase technique [27, 28]. Rabbit antibodies specific with respect to nuclear c-Fos protein (Oncogene Research, Ab-5, USA) and a standard ABC kit (Vector, PK4001, USA) were used. Labeled Fos-ir neurons in layers of the spinal gray matter were calculated using an optic microscope at magnifications ×250 and ×400; their localization was identified accordingly to the atlas [29].

The mean numbers of Fos-ir neurons ± s.e.m. (per slice) were calculated within layers 1-10 of the spinal gray matter bilaterally. A possible error related to double identification of one and the same labeled neuron in two neighboring slices was corrected using an Abercrombie equation [30]. Numerical data were analyzed using two-parameter dispersion analysis of variations (ANOVA); the *a posterior* Newman–Keuls criterion was additionally used.

RESULTS

Only single Fos-ir neurons (3-6 activated cells per slice) were observed in slices of the lumbar spinal segments of control rats (groups 1 and 2), and these units were localized exclusively in the dorsal horns. The numbers of Fos-ir neurons in the above spinal segments of rats of groups 1 and 2 were practically similar to each other. This fact shows that chloral hydrate anesthesia does not influence considerably the expression of gene *c-fos* in the absence of some special afferent and/or modulatory influences on neuronal networks of the above-mentioned spinal level.

After VS of the GS tendon in animals of group 3, activated neurons were observed in different layers of the gray matter in both ipsi- and contralateral slice halves with respect to the side of stimulation. Mean numbers of Fos-ir neurons in different segments, in general, increased in the rostro-caudal direction. The greatest concentration of labeled neurons was found in layers 4-7 of the L1-L2 segments (on average, 25.6 ± 1.4 units per slice). Fos-ir neurons were also detected in the imtermediolateral nuclei (INLs) of these segments (3.5 ± 0.5 cells). A noticeable number of labeled motoneurons was observed in layer 9 of the L4-L5 segments (6.9 ± 0.3 units; Fig. 1A, B, 2B, C, 3C). It should be noted that, accordingly to the atlas [29], layer 6 is absent in the gray matter of segments L1 and L2.

After isolated systemic injections of reserpine in the above-mentioned dose, rats of group 4 demonstrated typical modifications of the posture and motor activity. In such animals, we observed a flexor posture described earlier [31]. This posture was probably determined by intensification of contractions of the flexor muscles in the cervical and thoracic (trunk) regions and also of the forelimb flexors. Protraction in the rostral direction resulting from flexion in the coxal joints and extension in the knee and ankle joints was typical of the hindlimbs. Such postural modifications were accompanied by noticeable weakening of spontaneous and evoked motor activity of the animals (dyskinesia). The intensity of Fos-immunoreactivity in the spinal gray matter of rats of this group was greater as compared with that in the control (in rat groups 1 and 2), but, at the same time, this intensity was noticeably smaller than after isolated VS of the tendon in rats of group 3 (Fig. 1A, B, 2C, D). Most Fos-ir neurons in animals of group 4 were localized in the caudal segments of the lumbar spinal cord; activated motoneurons were also found at this level (Fig. 1C, D). It should be mentioned that, as compared with the pattern of Fos-immunoreactivity in group 3, about two-times greater mean number of labeled sympathetic preganglionic neurons (SPN) were detected in segments L1 and L2 of rats of group 4. Such neurons were observed in both ipsi- and contralateral IMLs (6.6 ± 0.5 and 7.2 ± 0.6 units, respectively; Fig 1C; 2E).

Vibration of the tendon in rats of group 5 performed 4 h after preliminary injections of reserpine provided higher intensity of Fos-immunoreactivity in the lumbar spinal segments. Values of the mean

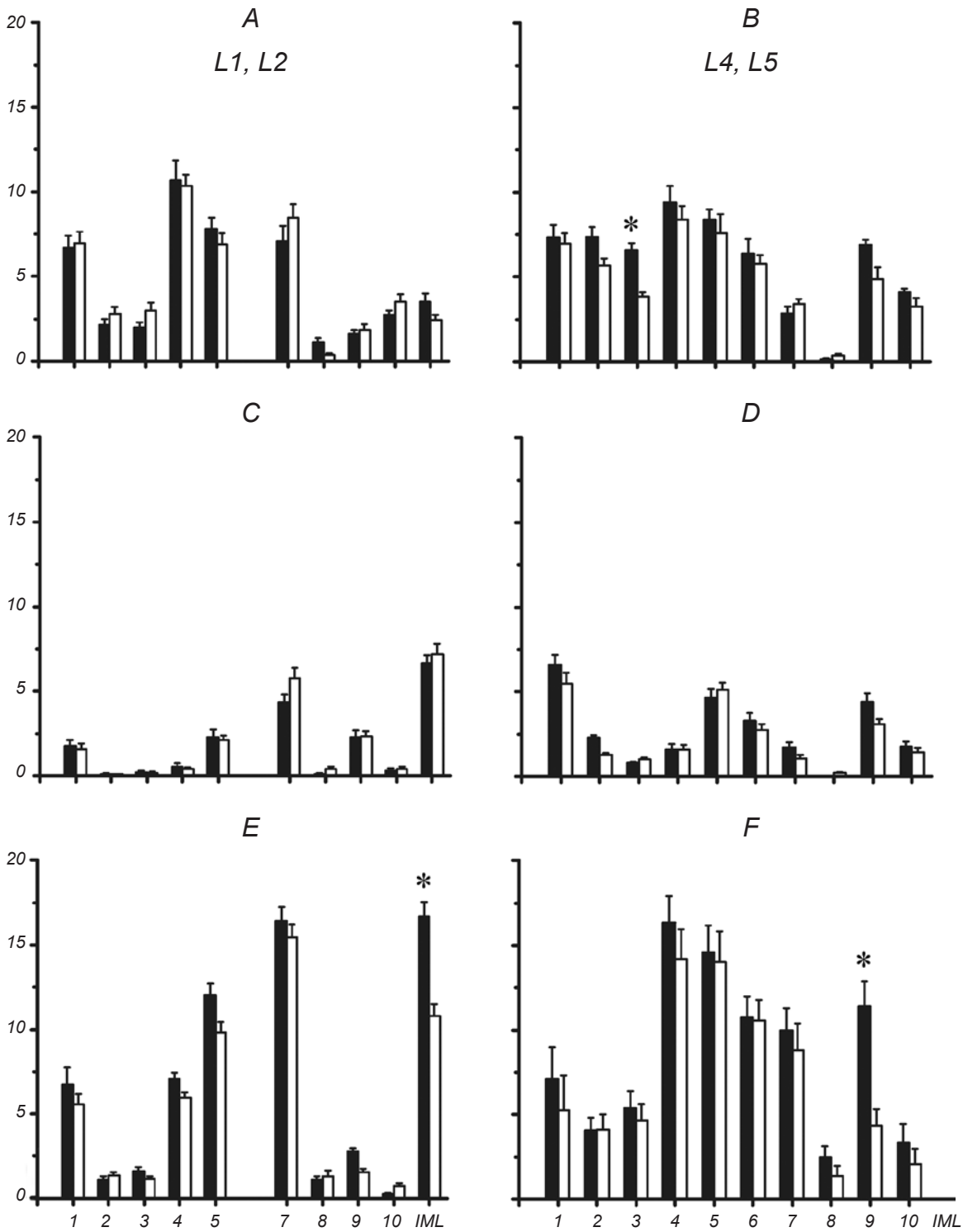

Fig. 1. Diagrams of the mean numbers (± s.e.m.) of Fos-ir neurons localized at the ipsilateral (filled columns) and contralateral (open columns) slice halves in layers 1-10 and in the intermediolateral nucleus (IML) of segments L1/L2 and L4/L5. A, B) After vibration of the *gastrecnemius+soleus* (GS) tendon; C, D) after i.p. introduction of reserpine; E, F) after vibration of the tendon combined with preliminary injections of reserpine. Asterisks above the columns show cases of significant differences between numbers of Fos-ir neurons within a layer in ipsi- and contralateral sides of the slice ($P < 0.01$).

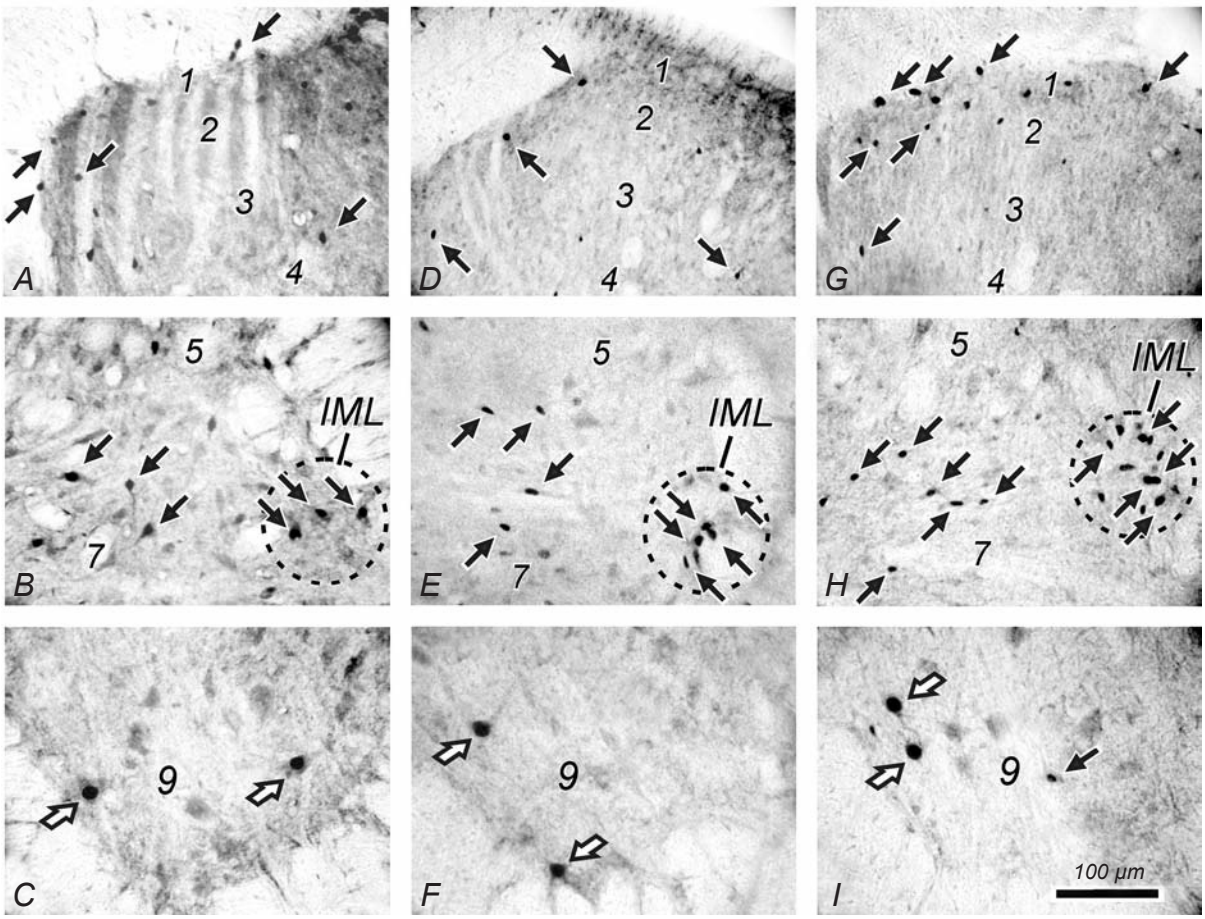

Fig. 2. Photomicrographs of frontal slices of the L1 segment (layers 1-9) of the rat spinal cord after vibrational stimulation of the GS tendon (A–C), injections of reserpine (D–F), and vibration of the tendon after preliminary injections of reserpine (G–I). Labeled nuclei of interneurons and motoneurons are shown by black and white arrows, respectively. Dashed lines in B, E, and H show borders of the intermediolateral nucleus (IML). Calibration 100 μm in I is similar for all fragments.

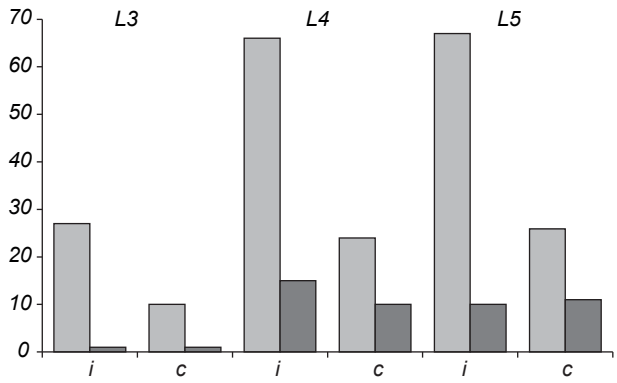

Fig. 3. Distiribution of Fos-immunoreactive (Fos-ir) motoneurons localized in different nuclei of layer 9. Labeled neurons were identified in 15 sections of each of the spinal segments L3, L4, and L5 after injections of reserpine and vibrational stimulation of the *mm. gastrocnemius* + *soleus*. Light and dark columns show numbers of Fos-ir motoneurons in the lateral and medial motor nuclei, respectively, ipsi- (i) and contralaterally (c) with respect to the side of stimulation.

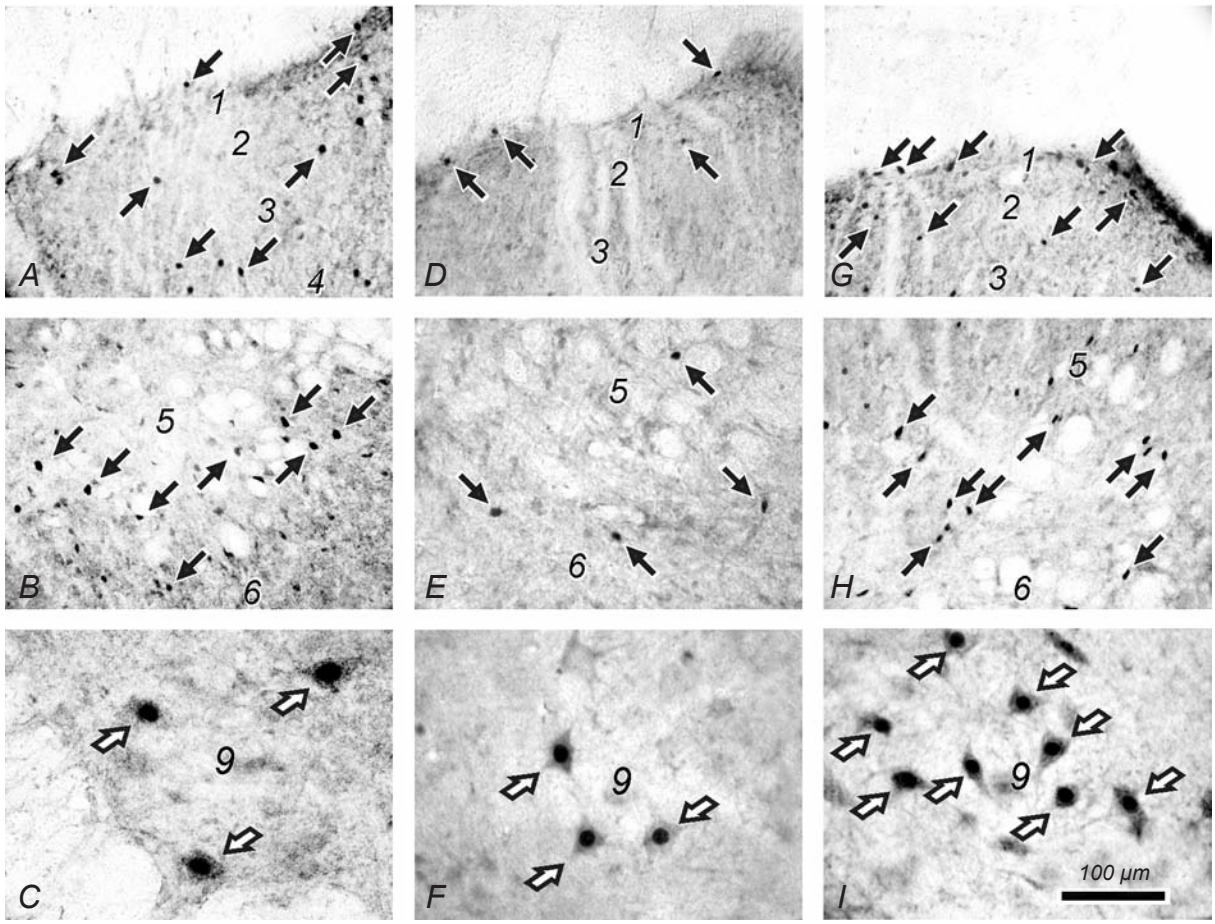

Fig. 4. Photomicrographs of frontal slices of the L4 segment (layers 1-9) of the rat spinal cord after vibrational stimulation of the GS tendon (A–C), injections of reserpine (D–F), and vibration of the tendon after preliminary injections of reserpine (G–I). Designations are similar to those in Fig. 2.

number of activated spinal neurons exceeded those observed in the same segments of animals of groups 3 and 4 (Fig. 1). It should be emphasized that large groupings of Fos-ir neurons were recorded in preliminarily reserpinized rats, bilaterally in layers 4-7 and ipsilaterally in the IMLs of segments L1 and L2 (35.4 ± 1.6 and 16.7 ± 0.9 units). In segments L4 and L5, this was observed in layers 4-7 and, additionally, in motor nuclei of layer 9 (51.7 ± ± 3.6 and 11.4 ± 1.5 of labeled units, respectively; Fig. 1E. G, 3I).

The main part of identified Fos-ir motoneurons was localized in segments L4 and L5. The distribution of labeled motoneurons calculated in 15 frontal 40-μm-thick slices of segments L3-L5 on ipsi- and contralateral sides with respect to the place of VS stimulation is shown in Fig. 3. Among 268 activated (according to the *c-fos* expression) motoneurons, 186 cells (69.4%) were localized ipsilaterally, while 82 cells (30.6%) were distributed contralaterally. Most ipsilateral Fos-ir motoneurons (160 cells, 86.0% of the ipsilateral population) were found in the lateral motor nuclei of layer 9, and only 26 units (14.0%) were found in the medial nuclei of this layer. The above nuclei (lateral and medial) in the mentioned regions of the contralateral halves of slices contained 60 (73.2%) and 22 (26.8%) labeled motoneurons, respectively.

DISCUSSION

Our comparative immunohistochemical study showed for the first time that increased levels of Fos-immunoreactivity in a few populations of segmental neurons of the lumbar spinal segments induced by VS of the GS tendon (in other words, increased levels of activation of the respective neuronal networks) demonstrate an additional increase after systemic application of reserpine. The greatest changes were manifested mostly in premotor neurons (layers 4-7) and motoneurons (layer 9) that are direct recipients of impulsation of group-1 and group-2 afferents from muscle stretch receptors; this was also found in SPNs of the intermediolateral spinal nuclei. As was found earlier, systemic injections of reserpine, the irreversible suppressor of vesicular transporter of monoamines, evokes a rapid and significant (down to 30% of the initial value) drop in the levels of biogenic amines (dopamine, noradrenaline, and serotonin) in structures of the brain and spinal cord [23, 32].

As is known, descending monoaminergic inputs to motoneurons of the spinal cord are diffuse; they can simultaneously influence motor pools of both agonists and antagonists and evoke co-activation of such muscle groups [33]. The formation of specific motor patterns (components of the motor repertoire) is realized with the necessary involvement of spinal inhibitory mechanisms, including systems of reciprocal inhibition. It is important to note that the formation of connections between monoaminergic systems and spinal cord within ontogenesis of mammals coincides with increase in the intensity of reciprocal inhibition and replacement of the synchronous type of activation of the limb muscles by an alternating one [34].

Pharmacological studies demonstrated that activation of the serotonergic and noradrenergic systems results in suppression of active behavior, while injuries of these systems result in motor hyperactivity [35, 36]. At the spinal level, monoamines facilitate synaptic transmission from group-1 afferents to 1a interneurons mediating reciprocal inhibition and also to 1a and 1b interneurons involved in realization of nonreciprocal inhibition. Simultaneously, these modulators suppress transmission of the influences from muscle afferents of group 2 [37]. Monoaminergic control of transmission of signals from muscle afferents is also realized via regulation of the presynaptic action of GABAergic interneurons [38] and direct effects of monoamines on gamma motoneurons [39]. As was recently found, the excitatory or inhibitory pattern of effects of some monoamines on one and the same neuron can significantly depend not only on the type of activated monoaminergic receptors but also on the site of localization of these receptors on one part of the membrane or another (somatic or dendritic) [40]. For example, serotonin can increase the excitability of motoneurons after activation of 5-HN2 serotonergic receptors and simultaneously decrease transmission of sensory effects on these cells via 5-HT1 receptors [41, 42]. Spatial distribution of receptors of these types on the membranes of target cells is differential.

Therefore, it can be supposed that intensification of the expression of gene *c-fos* found in our experiments in spinal premotor interneurons and motoneurons after application of reserpine is related, to a considerable extent, to weakening of inhibitory control of transmission of excitatory effects from muscle afferents provided by the descending monoaminergic systems. Such weakening of inhibition of interneurons in the pathways responsible for transmission of excitatory effects from muscle afferents to motoneurons can be interpreted as one of the factors determining the increased excitability of motoneurons and the respective development of rigidity/spasticity of the limb muscles observed in neurodegenerative diseases and after spinal trauma [14, 15, 43]. At present, the role of monoamines in the regulation of persistent Ca-dependent inward current in spinal motoneurons is attracting special attention as a factor responsible for the spasticity phenomenon [8, 14, 44].

Sympathetic preganglionic neurons integrate activity of the descending and sensory systems of autonomic control; these cells function as important centers of the formation of efferent signals of the sympathetic nervous system [45]. These neurons are localized mostly in the intermediolateral nuclei of the thoracolumbar part of the spinal cord; they receive main inputs from spinal interneurons and suprasegmental populations of sympathetic premotor neurons, including a large group of neurons in the rostral ventrolateral region of the medulla. Direct synaptic connections of primary afferents with SPNs are scarce. Nonetheless, a certain number of segmental sympathetic interneurons involved in the control of SPN activity were found in layers 5 and 7 of the spinal cord, i.e., within zones of projection of

muscle afferents [46, 47]. In the intact spinal cord, transsegmental connections with SPNs are mostly inactive; their activity begins to be manifested only after injuries of the descending (including monoaminergic) pathways resulting from trauma of the spinal cord [48].

Earlier studies demonstrated that monoamines influence the SPN excitability in a diverse manner; serotonin and noradrenaline increase the discharge frequency in most SPNs, but noradrenaline and dopamine are also capable of inhibiting these units [49]. The specific patterns of the modulatory actions of the above monoamines are related to activation of different types of monoaminergic receptors and different SPN subgroups having specific properties of their cellular membranes [50]. Our data on increase in the number of Fos-ir SPNs in the intermediolateral spinal nuclei of reserpinized rats show that preganglionic sympathetic activity was intensified under conditions of our experiments. These data agree with the findings that intravenous treatment with reserpine can enhance the background SPN activity. It is possible that such changes are realized due to suppression of supraspinal and/or segmental GABAergic control of the SPN activity [51, 52]. Decreases in the arterial pressure after the influence of reserpine was observed mostly in animals with an initially high level of the arterial pressure, even in the case were relatively large reserpine doses (5 mg/kg) were used. These effects were manifested only during a short period after applications of the above agent [25, 53, 54].

Thus, we can conclude that the activity of intraspinal neuronal networks activated via proprioceptive inputs at stimulation of muscle afferents is noticeably intensified under conditions of weakening of monoaminergic effects on spinal mechanisms after reserpine treatment, and the influences of such networks on motoneurons and SPNs increase in this case.

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