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Changes in Calbindin-Containing Neurons in the Posterior Horn of the Gray Matter of the Spinal Cord and the Sensory Ganglion of a Spinal Nerve in White Rats after Sensory Deprivation

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Immunohistochemical studies in Wistar rats ($n = 4$) addressed the effects of capsaicin on the morphometric and structural characteristics of 28-kDa calbindin (CB)-containing neurons in the posterior horn of the gray matter of segment T_{II} of the spinal cord (SC) and the second thoracic spinal nerve sensory ganglion (SNSG). Capsaicin was given to adult animals as three doses with intervals of 24 h, totaling 125 mg/kg, and specimens were collected on day 14. Administration of capsaicin decreased the proportion of CB-immunopositive (CB-IP) neurons in the SNSG (by 60%) and in laminae I, II, and III of the posterior horn (by 8%, 18%, and 15%, respectively); mean CB-IP neuron size increased as a result of intracellular edema. Deafferentation led to the development of consistent morphometric and structural changes in CB-IP neurons both in the SNSG and in the posterior horn of the gray matter of the SC, with central chromatolysis, and nuclear and cytoplasmic vacuolization, providing evidence of hydropic dystrophy. The irreversibility of these changes in SNSG neurons and laminae I, II and V of the posterior horn of the SC was evidenced by deformation of nuclei, lysis of nucleoli, and decreases in the numbers of CB-containing neurons, along with signs of neuronophagia, with the formation of residual nodules at the sites of dead cells.

Keywords: spinal cord, posterior horn, spinal nerve sensory ganglion, neurons, calbindin.

Primary sensory neurons bear TRPV1 receptors (transient receptor potential vanilloid 1 receptors), which interact with capsaicin, i.e., vanilloid derivatives [7, 12, 17, 19]. Capsaicin is a neurotoxin which at low doses has selective influences on most fine, non-myelinated afferent C fibers and, to some extent, fine, myelinated Aδ fibers. All the effects of capsaicin depend on the dose given and the duration of administration, so, depending on the aims of the study and the species and age of the animal, different capsaicin doses (from 50 to 150 mg/kg) and numbers of doses (single, repeated) are used. Low doses activate afferent terminals, producing an analgesic effect [11, 24]. Administration of single doses of capsaicin to neonatal animals produces a neurotoxic action [2, 3, 11]. Repeated administration of gradually increasing capsaicin doses induces desensitization, blockade of axoplasmic flow, depletion of neuropeptide reserves, and degeneration of sensory neurons [24].

Reactions to administration of a neurotoxic dose of capsaicin are reflected in functionally diverse neurons. Some authors have suggested that reactions induced by administration of capsaicin cannot be studied until two weeks from the moment of administration [6]. Others have claimed that the process of deafferentation is apparent by three days after giving capsaicin [7]. The neurotoxic action of capsaicin can also be explained by excessive intracellular accumulation of Ca^{2+} and Na^{+} ions, leading to activation of proteases and cell death [8,13].

Calbindin (CB) is an intracellular calcium-binding protein involved in transcellular transport of calcium ions which modulates the effects arising in response to changes in the intracellular calcium concentration, functioning as a characteristic buffer and supporting calcium homeostasis [18, 22].

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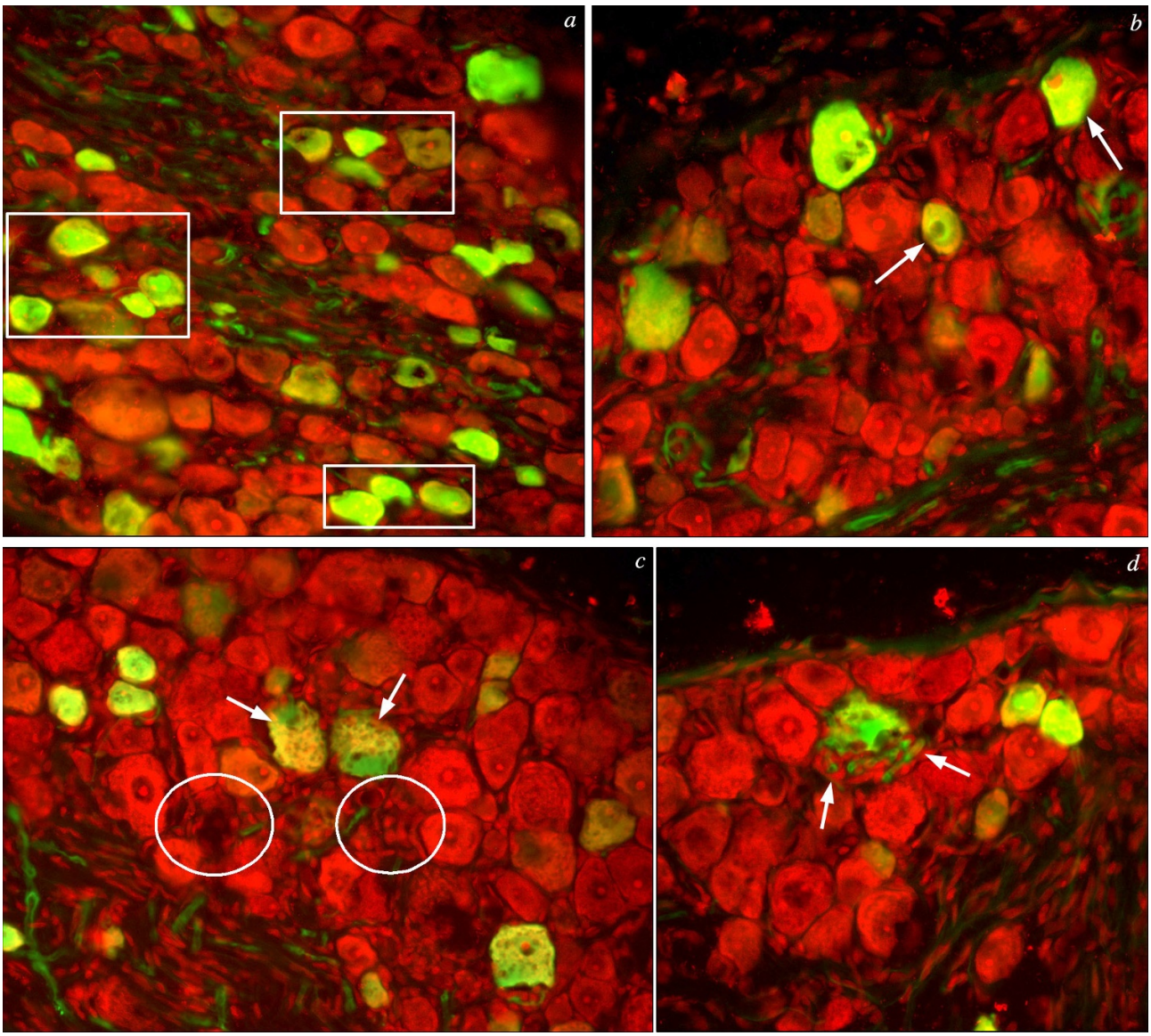

Fig. 1. Calbindin-immunopositive (CB-IP) neurons in the sensory ganglion of the second thoracic spinal nerve in control rats (*a*) and after administration of capsaicin (*b–d*). *a*) Clusters of CB-IP neurons (in boxes); *b*) cells with altered nuclei (arrows); *c*) cells with altered neuroplasm (arrows) and residual nodules of dead cells (circles); *d*) cells with clear glial reactions, neuronophagia (arrows). Immunohistochemical reactions. *a*, *b*) Objective ×10, ocular ×10; *c*, *d*) objective ×20, ocular ×10.

CB is present in different cell types in both the central [14, 16, 26] and peripheral [1, 4, 8, 15] nervous system. Dysfunction of the calcium buffer system in sensory neurons may result in degeneration of these cells [9]. Calcium-binding proteins have a special role in nerve cells, consisting of neuroprotection, which is related to their selective resistance to glutamate-induced neurotoxicity [20, 24].

The causes of stable changes to the neuron system of the posterior horn of the spinal cord (SC) due to injurious deafferentation is far from completely understood, though various mechanisms of sensitization have been identified. The neural networks of the posterior horn, forming functionally diverse modules, contain interneurons which are able to change in response to sensory information arriving via afferent A and C fibers [27].

The aim of the present work was to identify morphometric and structural changes to neurons containing 28-kD CB in the posterior horn of the SC and a spinal nerve sensory ganglion (SNSG) on the background of deficiency of afferentation induced by capsaicin.

Materials and Methods

Experiments were performed on female Wistar rats weighing 200 ± 10 g, which were divided into two groups: a control group (n = 4) and an experimental group (n = 4).

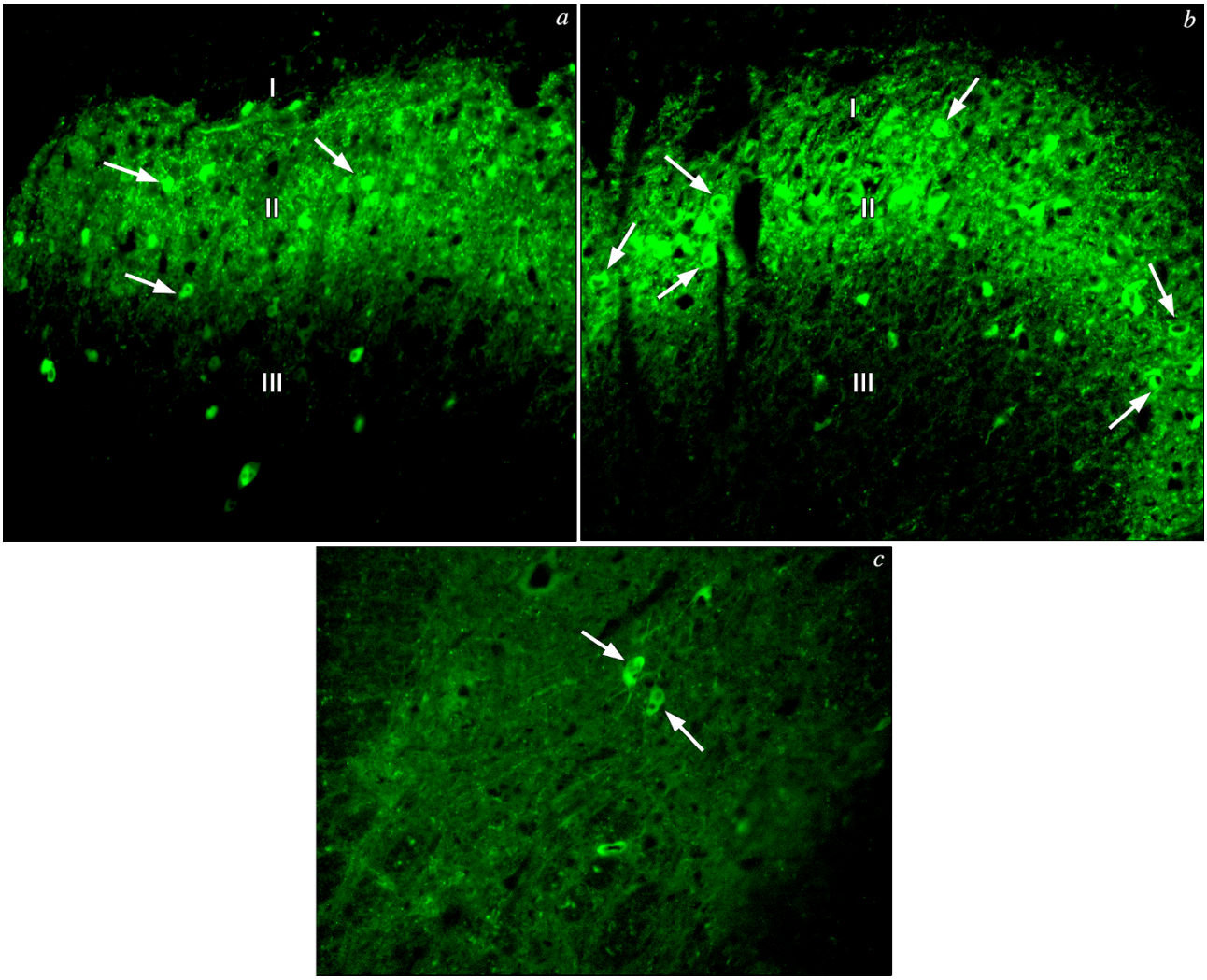

Fig. 2. Calbindin-immunopositive (CB-IP) interneurons in the posterior horn of the rat spinal cord in controls (*a*) and after administration of capsaicin (*b*, *c*). *a*) CB-IP interneurons (arrows); *b*) interneurons with CB-negative nuclei and narrow rims of cytoplasm (arrows); *c*) CB-IP interneurons with a "honeycomb" appearance (arrows); I, II, III – gray matter laminae (of Rexed). *a*, *b*) Objective ×20, ocular ×10; *c*) objective ×40, ocular ×10.

The experimental group was used to model deafferentation by s.c. administration of capsaicin (N-vanillylonanamide, Sigma) dissolved in 0.01 M phosphate-buffered saline (PBS) pH 7.4 (BioloT, Russia) containing 10% ethanol and 10% Tween-80. The total dose of capsaicin (125 mg/kg) was given to rats over three days: 25 mg/kg on day 1 and 50 mg/kg on days 2 and 3 [5]. Specimens were collected from animals of the experimental group on day 14 after the last capsaicin dose, simultaneously with collection of specimens from rats of the control group. All experimental procedures were performed in compliance with the "Regulations for Studies Using Experimental Animals." Euthanasia was performed under urethane anesthesia (3 g/kg, i.p.) by transcardiac perfusion with 0.01 M PBS pH 7.4 containing 5 U/liter heparin followed by 4% paraformaldehyde solution (Sigma, USA) in PBS. Experiments addressed posterior horn neurons in SC thoracic segment T_{II} and the second thoracic spinal nerve sensory ganglion. Isolated SC with roots and sensory ganglia were fixed in 4% paraformaldehyde in PBS for 2 h at 4°C, after which they were washed three times with PBS for 30 min and placed in 30% sucrose solution (Panreac, Spain) for 24 h at 4°C. T_{II} and SNSG were isolated from SC oriented in terms of roots and were frozen in Tissue-Tek O.C.T. Compound cryogel (Sakura Finetek, Holland). A Shandon E cryostat (Thermo Scientific, UK) was used to cut serial transverse sections of thickness 14 μm. Neurons were detected on each fifth section, thus using a total of 10 sections per specimen. CB-immunopositive (CB-IP) neurons were detected as described previously using labeled antibodies [1]: primary antibodies (Abcam, UK) were polyclonal rabbit anti-CB antibodies, diluted 1:500, and secondary antibodies were donkey anti-rabbit immunoglobulin G antibodies conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories, USA), di-

TABLE 1. Relative Contents (RC) of Calbindin-Immunopositive Neurons and Cross-Sectional Areas (S) in Rats in Controls and after Chemical Deafferentation with Capsaicin ($\bar{x} \pm s_{\bar{x}}$)

Group of animals	RC, %	S, μm²	Proportions of neurons of different size classes, %				
			≤200 μm²	201–400 μm²	401–600 μm²	601–800 μm²	>800 μm²
Control	26 ± 3	439 ± 27	9.7 ± 0.8	45.5 ± 0.6	28.3 ± 0.8	8.0 ± 0.4	8.5 ± 0.6
Experimental	10.3 ± 0.6*	552 ± 33*	6.0 ± 0.7*	27.5 ± 0.5*	35.5 ± 1.0*	15.3 ± 0.5*	15.7 ± 0.8*
*Significant differences compared with controls, $p < 0.05$.							

TABLE 2. Absolute Contents (AC) and Areas (S) of Calbindin-Immunopositive Interneurons in the Posterior Horn on Spinal Cord Sections ($\bar{x} \pm s_{\bar{x}}$)

Areas of gray matter of the spinal cord	Group of animals			
	Control		Experimental	
	AC	S, μm²	AC	S, μm²
Laminae of gray matter				
I	7.20 ± 0.18	54.8 ± 2.5	6.60 ± 0.22*	68 ± 5*
II	23.3 ± 1.1	46.2 ± 1.0	19.1 ± 1.2*	61.4 ± 1.7*
III–IV	8.1 ± 0.4	74 ± 8	6.90 ± 0.17*	80 ± 6
V	0.70 ± 0.23	147 ± 10	0.70 ± 0.24	187 ± 11*
Medial margin of posterior horn	1.0 ± 0.27	71.4 ± 2.5	0.80 ± 0.19	78.7 ± 1.8*
*Significant differences compared with controls, $p < 0.05$.				

luted 1:200, fluorescing in the green part of the spectrum. Labeling of the whole neuron population by the Nissl method was performed using NeuroTrace Red Fluorescent Nissl Stain (Molecular Probes, USA), which fluoresces in the red part of the spectrum, diluted 1:200, Sections were then washed with PBS and embedded in VectaShield medium for immunofluorescence (Vector Laboratories, USA). Controls to exclude nonspecific reactions were performed by incubating some sections in medium lacking primary antibody.

Specimens were examined under an Olympus BX43 microscope Olympus Corporation, Japan) fitted with the appropriate set of fluorescence blocking filters. Images were obtained using a cooled TCC-5.01CE digital video camera (Tucsen, China). A 10×/0.30 objective was used to study the topographic characteristics of interneurons in the posterior horn of the SC, determining their correspondence to Rexed laminae [21], whose configurations corresponded to the superior thoracic segments [23]. The cross-sectional areas of all IP neurons were measured using the program Image J (NIH, USA) on images of sections obtained using a 20×/0.50 objective. These same sections were used to count the numbers of IP neurons: on areas of 0.01 mm² in the SNSG and in each lamina of the gray matter of the SC in the dorsal horn. The proportion of IP neurons in the SNSG was taken as the ratio of the number of these cells to the total number of neurons, which was taken as 100%. SNSG neurons were characterized in terms of cross-sectional area using five size classes: less than 200 μm² (very small), 201–400 μm² (small), 401–600 μm² (intermediate), 601–800 μm² (large), and greater than 801 μm² (very large). Neurons whose sections passed through the nucleus and had visible nucleoli were used for the analysis. Arithmetic means and their standard errors were determined using Statistica 10 (StatSoft Inc., 2011). Differences in means were identified by analysis of variance (ANOVA) and were taken as significant at $p < 0.05$.

Results

The SNSG of both groups of rats showed CB-IP neurons and nerve fibers, while gliocytes were immunonegative. In controls, singly distributed and clusters of 2–8 cells of different sizes were distributed over the whole of ganglion sections (Fig. 1, *a*). The entire CB-IP neuron population had a green fluorescence, CB being distributed diffusely in the cytoplasm and, in some cells, in the nucleus. In terms of reaction product distribution, the CB-IP neuron population could be divided into three subpopulations: 1) consisting of small neurons with fluorescence more intense in the nucleus than in the cytoplasm; 2) consisting of large neurons with fluorescence more intense in the cytoplasm than in the nucleus; 3) consisting of neurons of different sizes with identically weak fluorescence in the nucleus and cytoplasm. Up to 30% of neurons in the SNSG contained CB. These were cells of all size classes: from very small to very large. About 50% were small neurons, and about 30% were of intermediate size. The remaining neurons were shared equally among IP cells of very small, large, and very large sizes (Table 1).

Administration of capsaicin was followed by detection of both singly distributed CB-IP neurons and clusters of these cells, including pairs, in sections of the SNSG, with 2–3 such clusters per section. Virtually all CB-IP neurons in the ganglion were of irregular shape and "honeycomb" appearance. Changes in some cells affected only the nucleus (see Fig. 1, *b*), which had cavities of different sizes and irregular outlines. Cavities in other cells filled not only the nucleus, but also the neuroplasm (see Fig. 1, *c*), without any perinuclear zone. Clear glial reactions were seen close to some of these cells (see Fig. 1, *d*), along with residual nodules (see Fig. 1, *c*). Levels of CB-IP neurons in the experiments decreased by 60% in the SNSG, though the mean size of cells of this population increased by 25% from the control size (see Table 1). Analysis of the cell composition of CB-IP neurons in the experimental group showed that the population consisted of cells of all size classes. Significant proportions of neurons were of small and intermediate sizes – 30% each – while 15% of cells were large and 15% were very large; the remaining small proportion in the ganglion consisted of very small neurons (see Table 1). This shows that chemical deafferentation altered the distribution of size groups, with a 1.6-fold decrease in the proportion of small neurons and a two-fold increase in the proportions of large and very large neurons.

In controls, the posterior horn of the SC contained CB-IP interneurons in all laminae – from I to V. The fluorescence intensity of IP neurons did not change in these laminae of the gray matter. CB-IP interneurons, which were round and showed fluorescence only in the cell body, were present in laminae I–II on every section. (Fig. 2, *a*).

Laminae III–IV on each section showed extended CB-IP interneurons on each section, the processes of these cells also fluorescing. The cell bodies were located along the dorsoventral axis, their process of length up to 20 μm propagating in the dorsal and ventral directions. Lamina V showed, on every fourth section, 1–4 CB-IP interneurons of triangular and extended shape, located in parallel with the dorsoventral axis, with fluorescing processes of length up to 10 μm running in the dorsal direction and process of length up to 20 μm running in the ventromedial direction. Throughout the medial margin (MM) of the posterior horn of the SC, every third section showed 3–4 CB-IP interneurons of round and extended shape with longer (up to 35 μm) fluorescing processes oriented in the dorsoventral direction.

In the T_{II} segment of the SC, the greatest number of CB-IP interneurons was found in lamina II (Table 2), with 3.2- and 2.9-fold smaller numbers in laminae I and III–IV respectively, and the smallest numbers in lamina V and the MM, where there were occasional CB-IP interneurons. The areas of CB-containing cells in the posterior horn of segment T_{II} ranged from 46.2 to 147 μm^2 (see Table 2). The largest interneurons were those in lamina V and the smallest were those in lamina II. Cells in laminae III–IV and the MM were of intermediate size, while CB-IP interneurons in lamina I were 18% larger than those in lamina II but 26% smaller than IP cells in laminae III–IV of the posterior horn.

After administration of capsaicin, CB-IP interneurons were detected in the same laminae of the posterior horn of the SC, and fluorescence in their processes was seen in laminae III–IV and V, and the MM. A characteristic structural feature of CB-IP interneurons in laminae I–II was provided by large CB-negative nuclei and the presence of a narrow rim of cytoplasm (Fig. 2, *b*). In lamina V, the bodies of CB-IP interneurons of "honeycomb" appearance contained cavities of different sizes located mainly in the nuclei and, in a small proportion of cells, in the nucleus and cytoplasm (see Fig. 2, *c*). Morphologically altered CB-IP interneurons were not seen in laminae III–IV and the MM. In segment T_{II} of the SC, the largest number of CB-IP interneurons was seen, as in controls, in lamina II (see Table 2), with 2.9 and 2.7 times fewer in laminae I and III–IV respectively and the smallest numbers in lamina V and the MM, where occasional cells were seen. The areas of CB-containing cells ranged from 61.4 to 187 μm^2 (see Table 2). The largest cells were interneurons in lamina V and the smallest were interneurons in lamina I. Cells in laminae III–IV and the MM were of intermediate size; CB-IP interneurons in lamina I were 10% larger than those in lamina II but 16% smaller than those in lamina III–IV. Capsaicin administration was followed by decreases in the numbers of IP cells in the laminae: by 8% in lamina I, 18% in lamina II, and 15% in laminae III–IV ($p < 0.05$). The mean size of IP interneurons, conversely, increased in virtually all laminae of the SC, where increases by were by 23% in lamina I, 33% in lamina II, 27% in lamina V, and 10% in the MM.

Discussion

This study established that the second thoracic SNSG in adult white rats contains neurons with CB immunoreactivity accounting for up to 30% of ganglion neurons, which is consistent with data reported from other studies [2, 4, 15]. This population consists of neurons of all size classes, from very small to very large; more than 50% of these IP neurons are of small (201–400 μm^2) and intermediate (401–600 μm^2) sizes. The sizes of CB-IP neurons in the SNSG are associated with their nociceptive functions [10, 24], as evidenced by the presence of CB in small peptidergic neurons [26]. In small TRPV1-containing neurons, CB is involved in chemosensory functions [12]. CB-IP interneurons in the gray matter of the posterior horn of the SC were located in all its laminae, more densely in lamina II, which is consistent with data reported by other investigators [4, 14]. The smallest CB-IP interneurons were cells in lamina II – with a mean size of no more than 50 μm^2, while the largest – with areas of greater than 100 μm^2 – were interneurons in lamina V.

Administration of the TRPV1 receptor agonist capsaicin led to a decrease in the proportion of CB-IP neurons in both the SNSG, by 60%, and in laminae I, II, and III of the posterior horn, by 8, 18, and 15%, respectively. Conversely, the mean cross-sectional area of CB-containing neurons in-

creased, due to intracellular edema: Nissl staining showed that capsaicin administration was followed by the development of central chromatolysis, with nuclear and cytoplasmic vacuolization, evidencing hydropic dystrophy of SNSG and posterior horn neurons. The irreversibility of these changes was indicated by nuclear deformation, nucleolar lysis, decreases in the numbers of CB-containing neurons, and signs of neuronophagia, leaving residual nodules at the locations of dead cells.

Like other studies [3, 25], the results obtained here provided evidence that administration of capsaicin was followed by neuron death in the SNSG, regardless of size; the mechanism of its harmful action was linked with intracellular accumulation of calcium ions resulting in the development of edema and subsequent cell destruction. The involvement of interneurons in laminae I, II, and V of the posterior horn in the destructive process may be explained by the cessation of transmission of information from primary sensory neurons [27]. The absence of nociceptive afferentation leads to the development of a state in which interneurons in the posterior horn of the SC perceive non-pain stimuli as nociceptive. This leads to the formation of central hypersensitivity (hyperalgesia) of interneurons in the posterior horn of the SC [6, 24], which is maintained by prolonged activation of posterior horn interneurons, especially in laminae I, II, and V, with release of glutamate, leading to the overaccumulation of calcium ions in postsynaptic neurons – with neurotoxic effects [20].

Thus, deafferentation leads to the development of consistent morphometric and structural changes to CB-IP neurons in both the SNSG and the posterior horn of the gray matter of the SC.

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