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Developmental Changes in NO Synthase-Containing Sensory Neurons in Chemical Deafferentation with Capsaicin

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Immunohistochemical and morphometric methods were used to determine the locations, relative contents, and morphometric characteristics of neurons expressing NO synthase (NOS) in the caudal ganglion of the vagus nerve (CGVN) and spinal nerve sensory ganglia (SNSG) in female Wistar rats ($n = 25$) aged 3, 10, 20, 30, and 60 days subjected to chemical deafferentation on the second day of life by administration of single doses of capsaicin. The control group consisted of rats ($n = 25$) of the same ages. The results obtained from these studies provided evidence that in the control group, the proportion of NOS-immunopositive neurons in the SNSG increased during the first 10 days of life and decreased from day 30 to day 60. In the CGVN, the proportion of NOS-immunopositive neurons did not show any significant change during ontogeny. In experimental animals, the proportions of NOS-positive neurons in the SNSG and CGVN decreased sharply during the first 20 days of life. The decrease in the number of NOS-containing neurons in the SNSG was more marked than that in the CGVN. These data provide evidence of the harmful effect of capsaicin on NOS-positive neurons, supporting the role of NO in the mechanisms of nociception.

Keywords: sensory ganglia, nitric oxide, ontogeny, immunohistochemistry.

Sensory ganglia contain a heterogeneous population of neurons [2–5, 9], including three groups: large neurons, small neurons containing neuropeptides, and small neurons not containing neuropeptides. Peptide-containing neurons are immunopositive for substance P or calcitonin gene-related peptide. Small neurons not containing peptides are able to bind *Griffonia simplicifolia* isolectin G4 (IB4) and are able to take part in a variety of pain syndromes, including post-traumatic [2, 9].

Studies of the mechanisms of pain in clinical and experimental investigations on laboratory animals make extensive use of capsaicin [3, 4, 10]. Neurophysiological and neurochemical studies at the end of the 1980s and 1990s showed that capsaicin, interacting with specific receptors on the plasmalemma, induces excitation and subsequent desensitization and morphological destruction in a large group of primary afferent C and some Aγ fibers. The neurotoxic action of capsaicin can be explained in terms of excessive intracellular accumulation of Ca^{2+} and Na^{+}, leading to hydration, activation of proteases, accumulation of toxins, and cell degeneration and death [9].

Treatment of neonatal rats with capsaicin has been shown to induce the death of 20% of cells in the thoracic ganglion of the spinal nerve on day 10 of life. This involves the death of the population of small neurons, these being capsaicin-sensitive and containing TRPV1 receptors. In older animals, the proportion of TRPV1-immunopositive neurons in the ganglion was also decreased by 12–20% [3, 4, 6, 8].

Neurotransmitters include small molecules, particularly nitric oxide (NO). Neurons containing NO synthase (NOS) are detected in the sensory ganglia in rats, particular-

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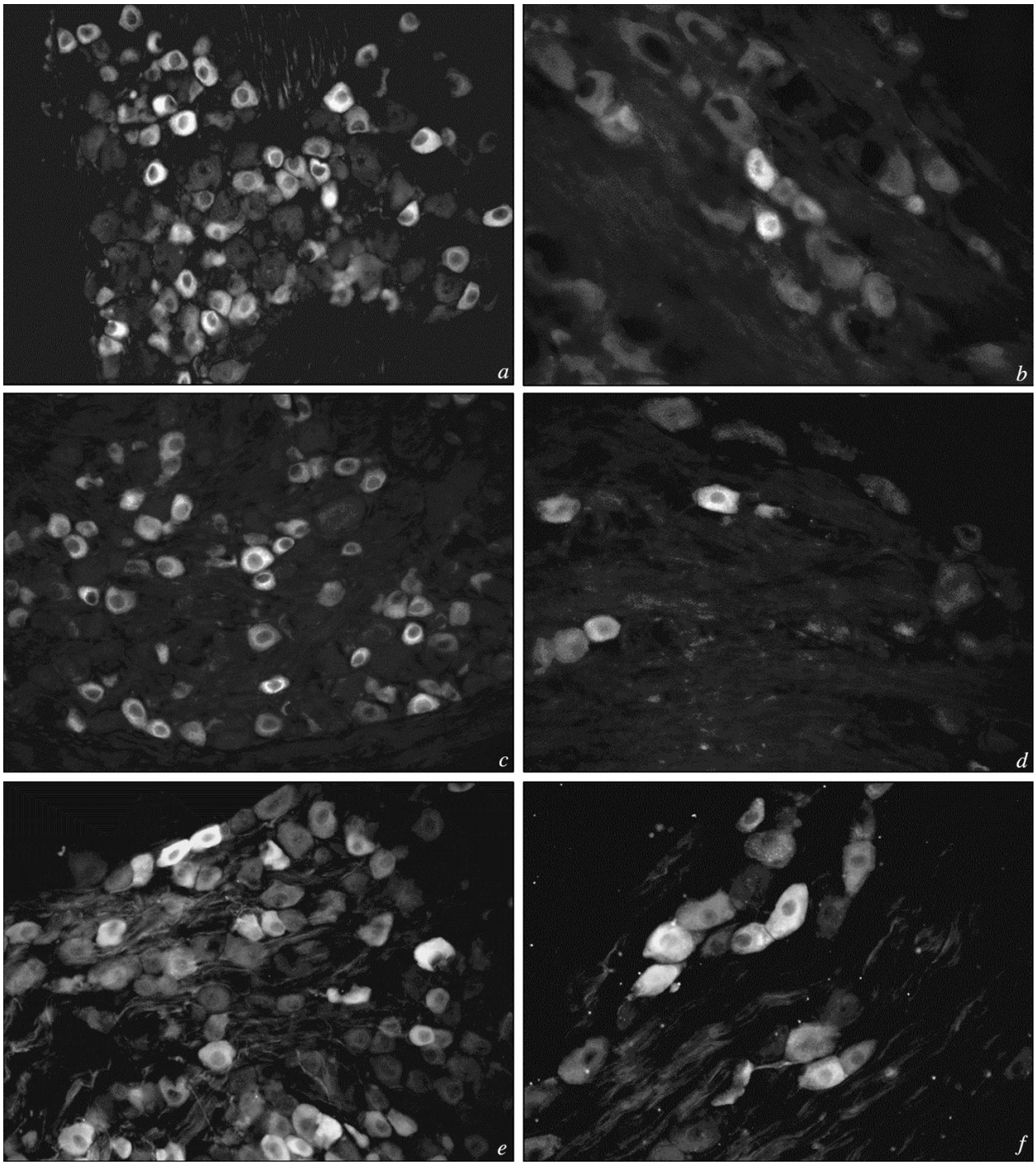

Fig. 1. NOS synthase-containing neurons in the second thoracic (T_{II}) spinal nerve sensory ganglion (SNSG) (*a*, *b*), the fourth lumbar (L_{IV}) SNSG (*c*, *d*), and the caudal nucleus of the vagus nerve (*e*, *f*) in rats aged 10 days (*a*, *c*, *e*) and 60 days (*b*, *d*, *f*) after chemical deafferentation with capsaicin. Immunohistochemical reaction with fluorescein isothiocyanate. Objective ×10, ocular ×10.

ly in the caudal ganglion of the vagus nerve (CGVN) and the spinal nerve sensory ganglia (SNSG). The proportion of NOS-positive neurons changes during postnatal ontogeny. During the first 10 days of life, the SNSG shows an increase in the proportion of NOS-positive neurons, with a subsequent decrease between day 30 and day 60. In the CGVN, the proportion of NOS-immunopositive neurons does not show any significant change during ontogeny [9].

The literature lacks reports of the influence of chemical deafferentation on NOS-containing neurons. The aim of the

TABLE 1. Relative Contents of NOS-Immunopositive Neurons in the Spinal Nerve Sensory Ganglia (SNSG) and the Caudal Ganglia of the Vagus Nerve (CGVN) in Control Rats and after Administration of Capsaicin ($\bar{x} \pm s_{\bar{x}}$, %)

Group of animals	Age, days	T_{II} SNSG	L_{IV} SNSG	CGVN
Control	3	41.7 ± 2.8	41 ± 3	35.1 ± 2.2**
	10	55.4 ± 2.5*	54.6 ± 2.1*	37.1 ± 1.8**
	20	63 ± 3*	60 ± 3*	41.3 ± 2.2**
	30	63 ± 3*	60.6 ± 2.1*	40.5 ± 2.4**
	60	52.9 ± 2.4*	50.9 ± 2.4*	39.2 ± 2.5**
Experimental	3	42 ± 3	40 ± 4	32.7 ± 1.8
	10	36.9 ± 2.5*	26 ± 3*	15.0 ± 2.7*.**
	20	9.9 ± 1.7*	10.1 ± 2.5*	11.8 ± 2.9*
	30	5.3 ± 0.9*	9.8 ± 1.6*	18 ± 3*.**
	60	7.8 ± 1.4*	11.9 ± 1.6*	24 ± 3*.**

*Significant differences compared with values in three-day-old rats, $p < 0.05$; **significant differences compared with T_{II} and L_{IV} SNSG, $p < 0.05$.

TABLE 2. Cross-Sectional Areas of NOS-Immunopositive (NOS^+) and NOS-Immunonegative (NOS^-) Neurons in the Spinal Nerve Sensory Ganglia (SNSG) and the Caudal Ganglia of the Vagus Nerve (CGVN) in Control Rats and after Chemical Deafferentation with Capsaicin ($\bar{x} \pm s_{\bar{x}}$, μm^2)

Group of animals	Age, days	T_{II} SNSG		L_{IV} SNSG		CGVN	
		NOS^+	NOS^-	NOS^+	NOS^-	NOS^+	NOS^-
Control	3	239 ± 13	261 ± 11	245 ± 16	238 ± 12	227 ± 6	222 ± 6
	10	365 ± 10*	448 ± 15	329 ± 6*	448 ± 12	357 ± 14	328 ± 12
	20	381 ± 14*	504 ± 14	337 ± 13*	564 ± 34	421 ± 24	381 ± 11
	30	405 ± 13*	520 ± 21	382 ± 12*	557 ± 20	468 ± 19	452 ± 10
	60	469 ± 10*	576 ± 34	483 ± 12*	541 ± 12	502 ± 19	485 ± 10
Experimental	3	239 ± 13	261 ± 11	245 ± 16	238 ± 12	227 ± 6	222 ± 6
	10	360 ± 7*	450 ± 16	354 ± 12*	432 ± 12	381 ± 14*	324 ± 6
	20	378 ± 4*	523 ± 14	442 ± 34*	598 ± 26	584 ± 39	560 ± 14
	30	561 ± 16*	618 ± 22	526 ± 22*	743 ± 27	550 ± 33	533 ± 18
	60	508 ± 14*	553 ± 20	489 ± 31*	627 ± 40	542 ± 24*	531 ± 16

*Significant differences compared with NOS^-, $p < 0.05$.

present work was therefore to identify the locations, relative contents, and morphometric characteristics of NOS-immunopositive neurons in the SNSG and CGVN in chemical deafferentation evoked by capsaicin during postnatal ontogeny.

Materials and Methods

Experiments were performed on 50 white male Wistar rats aged 3, 10, 20, 30, and 60 days in compliance with the "Regulations for Studies Using Experimental Animals" (USSR Ministry of Health Decree No. 755 of August 12, 1977). The animals were divided into two groups: a control group ($n = 25$) and an experimental group ($n = 25$). Rats of the experimental group underwent deafferentation on day 2 of life by administration of single s.c. doses of capsaicin (N-vanillylonanamide, Sigma, USA), 100 mg/kg, in solution consisting of one part of 96% ethanol, one part of tween-80, and eight parts of isotonic NaCl solution. Animals were subjected to euthanasia under urethane anesthesia (3 g/kg, i.p.) by transcardiac perfusion with phosphate-buffered saline (PBS) containing heparin (5 U/liter) followed by 4% paraformaldehyde in 0.1 M PBS. The investigations used the sensory ganglion of the spinal nerves of the second thoracic (SNSG T_{II}) and the fourth lumbar (SNSG L_{IV}) segments, as well as the CGVN. Isolated ganglia were placed

in fixative for 2 h and were then washed three times with PBS (30 min each wash) and placed in 30% sucrose solution for 24 h. Serial sections of thickness 14 μm were cut from the fixed material using a cryostat.

NOS-containing neurons were detected using labeled goat antibodies (Abcam, USA) at a dilution of 1:300. Sections were preincubated for 30 min at room temperature in PBS supplemented with 10% serum, 1% Triton X-100, 0.1% bovine serum albumin, and 0.05% thiomersal. Sections were then incubated with primary antibodies for 24 h at room temperature. After brief washing with PBS, sections were incubated with secondary antibodies conjugated with fluorescein isothiocyanate (FITC), which gives a green fluorescence (Jackson, USA, diluted 1:100) for 2 h.

Proportions of immunopositive neurons were determined by labeling all neurons in the population with the fluorochrome NeuroTrace Red (Molecular Probes, USA), with a red fluorescence. After washing with PBS, sections were incubated in this solution for 20 min (diluted 1:200). Sections were then washed with PBS and embedded in medium for immunofluorescence (VectaShield, Vector Laboratories, USA).

Sections were analyzed using an Olympus BX43 fluorescence microscope (Olympus, Japan) with the appropriate set of light filters and a Tucsen TCH 5.0 cooled CCD digital camera (Tucsen, China) and ISCapture 3.6 (Tucsen, China). The sizes and relative contents of immunopositive neurons were studied using the three central sections of each ganglion, cut with intervals of about 0.05 mm. Counts included only neurons with clearly identifiable nuclei. The proportion of immunopositive neurons was taken as the ratio of these cells to the total number of neurons on the section, which was taken as 100%. Cross-sectional areas of neurons were measured using ImageJ (NIH, USA). This value was determined in a random selection of using the method described by Avtandilov [1]; 100 neurons immunopositive for each of the markers were evaluated in each age group.

Statistical analysis included calculation of the arithmetic mean and its standard error. Significant differences were identified using Student's *t* test and were regarded as significant at $p < 0.05$.

Results

NOS-positive neurons were detected in both the SNSG and the CGVN in all age groups in the control and experimental groups (Fig. 1). In animals of the control group, the proportion of NOS-immunopositive neurons in the SNSG increased during the first ten days of life and decreased during the period from day 30 to day 60. In the CGVN, the proportion of NOS-immunopositive neurons showed no significant change during ontogeny ($p > 0.05$, Table 1). In each age group, the proportion of NOS-immunopositive neurons in the CGVN was significantly smaller than that in the SNSG. There were no significant differences in the proportions of NOS-immunopositive neurons between the SNSG of the T_{II} and L_{IV} segments in the control group ($p > 0.05$).

In animals of the experimental group, the proportions of NOS-positive neurons in the SNSG and CGVN decreased sharply during the first 20 days of life (see Table 1). There were no significant differences in the proportions of NOS-immunopositive neurons in the SNSG of the T_{II} and L_{IV} segments, with the exception of age 10 days, when the proportion of immunopositive neurons was significantly greater in the T_{II} SNSG ($p < 0.05$). A greater reduction in the number of NOS-containing neurons was seen in the SNSG than the CGVN. In the CGVN, by the end of the second month of life, the proportion of NOS-immunopositive neurons increased, but nonetheless remained significantly lower than in control animals ($p < 0.01$) (see Table 1).

The mean cross-sectional area of NOS-immunopositive and immunonegative cells increased during ontogeny in all the ganglia studied from the moment of birth to the second month of life in the control group and one month of life in the experimental group (Table 2). In both groups, from day 10 of life, the mean cross-sectional area of NOS-immunopositive neurons in the SNSG became significantly smaller than that of NOS-immunonegative neurons ($p < 0.05$). The mean cross-sectional areas of NOS-positive and immunonegative neurons in the SNSG and CGVN in experimental 30-day-old rats were significantly greater than that those in control rats ($p < 0.05$).

Discussion

This study established that during early postnatal ontogeny, the proportion of NOS-immunopositive neurons in the SNSG control rats changed, reaching a maximum on day 30 of life, which is consistent with previously published results [9].

Chemical deafferentation due to capsaicin produced a significant reduction in the proportion of NOS-positive neurons during the first 20 days of life. However, in the CGVN, the proportion of NOS-immunopositive neurons again increased after 20 days, though it remained lower than in animals of the control group. It is possible that the visceral afferent neurons in the CGVN are more resistant to chemical deafferentation than those in the SNSG, where there are many somatic afferent neurons.

Chemical deafferentation with capsaicin significantly increased the mean cross-sectional areas of NOS-positive and -negative neurons in experimental rats on day 30 of life, as compared with controls. This may be explained by the death of the smallest neurons, which are the most sensitive to capsaicin [3, 4, 8], and by increases in neuron size due to edema resulting from the toxic action of this neurotoxin. Neuron size slowly recovered by the end of the second month of life, such that they again had morphological parameters comparable to those of control rats.

Quite contradictory data have been obtained providing evidence of the pronociceptive and antinociceptive actions of NO in sensory neurons [11]. Many data provide evidence of the role of NO in the development of sensitization in inflammatory and neuropathic pain [7, 11]. NO may play a

role in the processes of sensitization due to cGMP synthesis and subsequent phosphorylation of specific membrane proteins, mediated via protein kinase C. NO may also activate different types of TRP channels, including TRPC5, TRPV1, TRPV3, TRPV4, and TRPA1 [11, 12].

Thus, the data obtained here provide evidence that capsaicin has harmful actions on NOS-positive neurons, which supports the role of NO in the mechanisms of nociception. NOS-immunopositive CNS in the SNSG are more susceptible to the toxic effect of capsaicin than those in the CGVN.

REFERENCES

1. G. G. Avtandilov, *Medical Morphometry*, Meditsina, Moscow (1990).
2. P. M. Maslyukov, V. V. Porseva, M. B. Korzina, and A. D. Nozdrachev, "Neurochemical features of sensory neurons in ontogeny," *Ros. Fiziol. Zh.*, **99**, No. 7, 777–792 (2013).
3. V. V. Porseva, V. V. Shilkin, M. B. Korzina, et al., "Changes in TRPV1-immunoreactive neurons in rat spinal nerve sensory ganglia in response to capsaicin," *Morfologiya*, **139**, No. 3, 41–45 (2011).
4. V. V. Porseva, A. A. Strelkov, V. V. Shilkin, and P. M. Maslyukov, "Developmental changes in calcitonin gene-related peptide-containing sensory neurons in conditions of deficient afferentation in rats," *Ontogenez*, **43**, No. 6, 405–412 (2012).
5. I. S. Raginov and Yu. A. Chelyshev, "Post-traumatic survival of sensory neurons of different subpopulations," *Morfologiya*, **124**, No. 4, 47–50 (2003).
6. Z. R. Gallaher, R. M. Larios, V. Ryu, et al., "Recovery of viscerosensory innervation from the dorsal root ganglia of the adult rat following capsaicin-induced injury," *J. Comp. Neurol.*, **518**, No. 17, 3529–3540 (2010).
7. W. Kallenborn-Gerhardt, K. Schröder, G. Geisslinter, and A. Schmidtko, "NOXious signaling in pain processing," *Pharmacol. Ther.*, **137**, 309–317 (2013).
8. Q. P. Ma, "Expression of capsaicin receptor (VR1) by myelinated primary afferent neurons in rats," *J. Neurosci. Lett.*, **319**, 87–90 (2002).
9. P. M. Masliukov, A. I. Emanuilov, L. C. Madalieva, et al., "Development of nNOS-positive neurons in the rat sensory and sympathetic ganglia," *Neuroscience*, **256**, 271–281 (2014).
10. L. Pecze, W. Blum, and B. Schwaller, "Mechanism of capsaicin receptor TRPV1-mediated toxicity in pain-sensing neurons focusing on the effects of Na(+)/Ca(2+) fluxes and the Ca(2+)-binding protein calretinin," *Biochem. Biophys. Acta* **1833**, No. 7, 1680–1691 (2013).
11. G. Petho and P. W. Reeh, "Sensory and signaling mechanisms of bradykinin, eicosanoids, platelet-activating factor, and nitric oxide in peripheral nociceptors," *Physiol. Rev.*, **92**, 1699–1775 (2012).
12. T. Yoshida, R. Inoue, T. Morii, et al., "Nitric oxide activates TRP channels by cysteine S-nitrosylation," *Nat. Chem. Biol.*, **2**, 596–607 (2006).