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Developmental Changes in NO-Containing Sympathetic Neurons in the Spinal Cord in Rats

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Objectives. To determine the locations, percentage composition, and morphometric characteristics of sympathetic preganglionic neurons containing NO synthase (NOS) in the spinal cord of rats. **Materials and methods.** Experiments were performed on 35 white female Wistar rats aged 3, 10, 20, 30, and 60 days, 6 months, and 3 years. Immunohistochemical detection of NOS and the acetylcholine synthesis enzyme choline acetyltransferase (CAT) was performed on sections of spinal cord made at the level of segment T2. The areas of nerve cells and the proportions of immunoreactive neurons were determined. **Results.** Most sympathetic preganglionic neurons in the spinal cord of neonates and 10-day-old rats contained both NOS and CAT. At these age groups, rats also had a population of NOS-positive/CAT-negative neurons (26% in neonates and 8% in 10-day-old animals), this population was not seen in older animals. During the first 20 days, the proportion of NOS-immunopositive neurons decreased significantly, while the population of CAT-positive neurons increased. **Conclusions.** There was a reduction in the number of sympathetic preganglionic neurons expressing NOS during early postnatal ontogeny, which may influence the mechanisms of NO-ergic sympathetic transmission.

Keywords: sympathetic preganglionic neurons, NO synthase, spinal cord, immunohistochemistry, rats.

Sympathetic preganglionic neurons are present in the spinal cord not only in the lateral horn, where the greater part of the intermediolateral nucleus (nucleus intermediolateralis, pars principalis, nucl. ILp) and the funicular part of this nucleus (nucleus intermediolateralis, pars funicularis – nucl. ILf) are located, and also in the dorsally located intercalated spinal nucleus (nucleus intercalatus spinalis – nucl. IC), including its paraependymal part (nucleus intercalatus spinalis, pars paraependymalis – nucl. ICpe) [5]. Neurons in these nuclei are cholinergic and contain acetylcholine, which is synthesized by choline acetyltransferase (CAT), the key acetylcholine synthesis enzyme. Along with CAT, NO synthase (NOS) is also present in cholinergic neurons; this enzyme synthesizes nitric oxide [14].

Nitric oxide (NO) functions as a universal modulator of a variety of functions in the body, including the control of respiration and circulation, maintenance of the body's immune status, nervous tissue plasticity, memory, and neurotransmitter release [3, 4, 12]. NO is an intra- and intercellular mediator, carrying out a variety of signal functions. In synapses, NO can act at the pre- and postsynaptic levels [3, 8, 12]. The neurotransmitter composition of neurons in the autonomic nervous system, particularly sympathetic ganglia, changes during postnatal ontogeny [2]. Current published data provide evidence that NOS and CAT can be colocated in individual neurons in the spinal cord from the moment of birth [1]. However, there are few data on developmental changes in the neurochemical composition of preganglionic sympathetic neurons. Therefore, the aim of the present work was to identify the locations, percentage composition, and morphological characteristics of preganglionic sympathetic NOS-immunoreactive neurons in the spinal cord during postnatal ontogeny.

Materials and Methods. Studies were carried out on 35 female Wistar rats aged 3, 10, 20, 30, and 60 days, 6 months, and 3 years from birth. Keeping, experiments, and

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TABLE 1. Relative Content of NOS-Positive Neurons in Rat Spinal Cord Nuclei ($\bar{x} \pm s_{\bar{x}}$, %)

Age	nucl. ILf	nucl. ILp	nucl. IC	nucl. ICpe
Neonatal	9 ± 0.4	56 ± 3.4	14 ± 1.9	19 ± 2.1
10 days	11 ± 0.6	54 ± 2.7	15 ± 1.5	18 ± 1.8
20 days	12 ± 0.8	60 ± 4.3	12 ± 1.8	15 ± 1.6
30 days	10 ± 0.5	59 ± 3.5	13 ± 1.1	16 ± 1.7
2 months	14 ± 1.1*	57 ± 3.9	15 ± 1.9	12 ± 1.8*
6 months	14 ± 0.6*	56 ± 3.8	16 ± 1.2**	13 ± 1.4*
3 years	14 ± 0.5*	56 ± 4.1	17 ± 1.1**	12 ± 1.6*

* Significant difference compared with neonates, $p < 0.05$. ** Significant differences compared with 20-day-old rats, $p < 0.05$.

harvesting of animals were carried out in compliance with the "Regulations for Studies Using Experimental Animals." This study was approved by the Ethics Committee (No. 11, of September 29, 2016). Euthanasia was performed under urethane anesthesia (3 g/kg, i.p.) by transcardiac perfusion with isotonic sodium chloride solution containing heparin (5 U/liter) followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Studies used thoracic spinal segment T2. Materials were fixed for 2 h in the same paraformaldehyde solution and then washed three times in 0.01 M phosphate-buffered saline (PBS) pH 7.4 (Biolot Ltd., St. Petersburg, Russia) for 30 min and left in 30% sucrose solution for 24 h. Serial sections of thickness 14 μm were prepared from the fixed specimens using a cryostat.

NOS-immunoreactive neurons were detected by labeling with polyclonal rabbit antibodies to the neuronal isoform of NOS from LifeSpan Biosciences (USA), cat. No. LS-B8696, at a dilution of 1:300, and antibodies to CAT, goat antibodies from Millipore (USA), at a dilution of 1:50. Sections were preincubated for 30 min at room temperature in PBS containing 10% donkey serum (Jackson Immunoresearch, USA), 1% Triton X-100 (Sigma-Aldrich, USA), 0.1% bovine serum albumin (Biolot Ltd., St. Petersburg, Russia), and 0.05% thimerosal. Sections were then incubated with primary antibodies for 24 h at room temperature. After brief washing in PBS, sections were incubated with secondary donkey antirabbit and antigoat antibodies conjugated with the fluorochromes FITC or Cy3 (Jackson Immunoresearch, USA for 2 h (dilution 1:150), giving green or red fluorescence. Sections were then washed with PBS and embedded in medium for immunofluorescence (VectaShield, Vector Laboratories, USA).

Preparations subjected to the immunohistochemical processing procedure were analyzed using a programmable system including an Olympus BX45 fluorescence microscope (Japan) with a set of filters (mirror module U-FBWA – blue excitation, excitation filter BP460-495, barrier filter BA510-550; mirror module U-FGWA – green excitation, excitation filter BP530-550, barrier filter BA575-625), a Tucsen TCH-5.0ICE cooled CCD camera (Xintu Photonics, China), and a computer with an Intel Core i7 processor. Image acquisition and subsequent processing were performed using ISCapture version 3.6 (Xintu Photonics, China). The morphological properties of neurons (circularity coefficient, cross-sectional area, maximum diameter) were determined using ImageJ (NIH, USA). Areas of nerve cells and the percentages of immunoreactive neurons were determined. Analysis was confined to those neve cells in which the section passed through the nucleus.

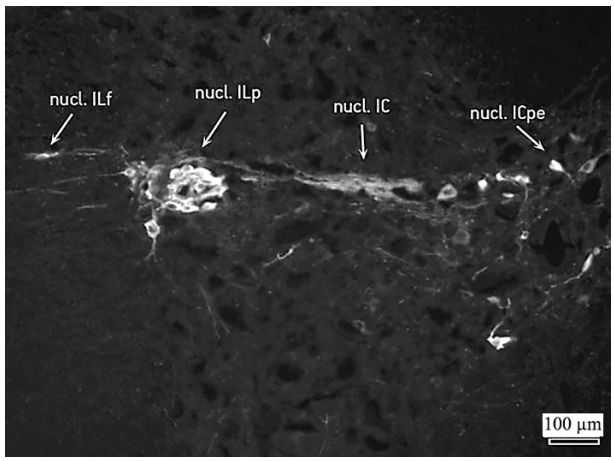

Fig. 1. NOS-immunoreactive neurons in 20-day-old rats in the autonomic nuclei of the rat spinal cord. Fluorescence, Cy3.

The proportions of NOS-immunopositive/CAT-immunonegative and NOS-immunonegative/CAT-immunopositive neurons were determined. The ratios of each of these groups of cells to the total number (NOS + CAT) of immunopositive neurons, which was taken as 100%, were determined. Cross-sectional areas of neurons were measured by selecting 100 NOS-immunopositive neurons from each age group in random order.

Statistical analysis included computation of the arithmetic mean and its standard error. Values were compared

TABLE 2. Relative Contents of CAT- and NOS-Immunoreactive Neurons in the ILp Nucleus during Ontogeny ($\bar{x} \pm s_{\bar{x}}$, %)

Age	NOS(+)/CAT(–)	NOS(+)/CAT(+)	NOS(–)/CAT(–)
Neonatal	26 ± 4.5**	66 ± 5.8	8 ± 2.3**
10 days	8 ± 3.2*	75 ± 4.6	17 ± 3.3*
20 days	<1*,**	76 ± 3.8	23 ± 2.1*
30 days	<1*,**	55 ± 3.3**	45 ± 3.6*,**
2 months	<1*,**	59 ± 4.2**	41 ± 2.2*,**
6 months	<1*,**	62 ± 5.1**	38 ± 4.5*,**
3 years	<1*,**	56 ± 4.6**	44 ± 3.7*,**

* Significant difference compared with neonates, $p < 0.01$. ** Significant differences compared with 10-day-old rats, $p < 0.05$. The total number of CAT- and NOS-immunopositive neurons was taken as 100%.

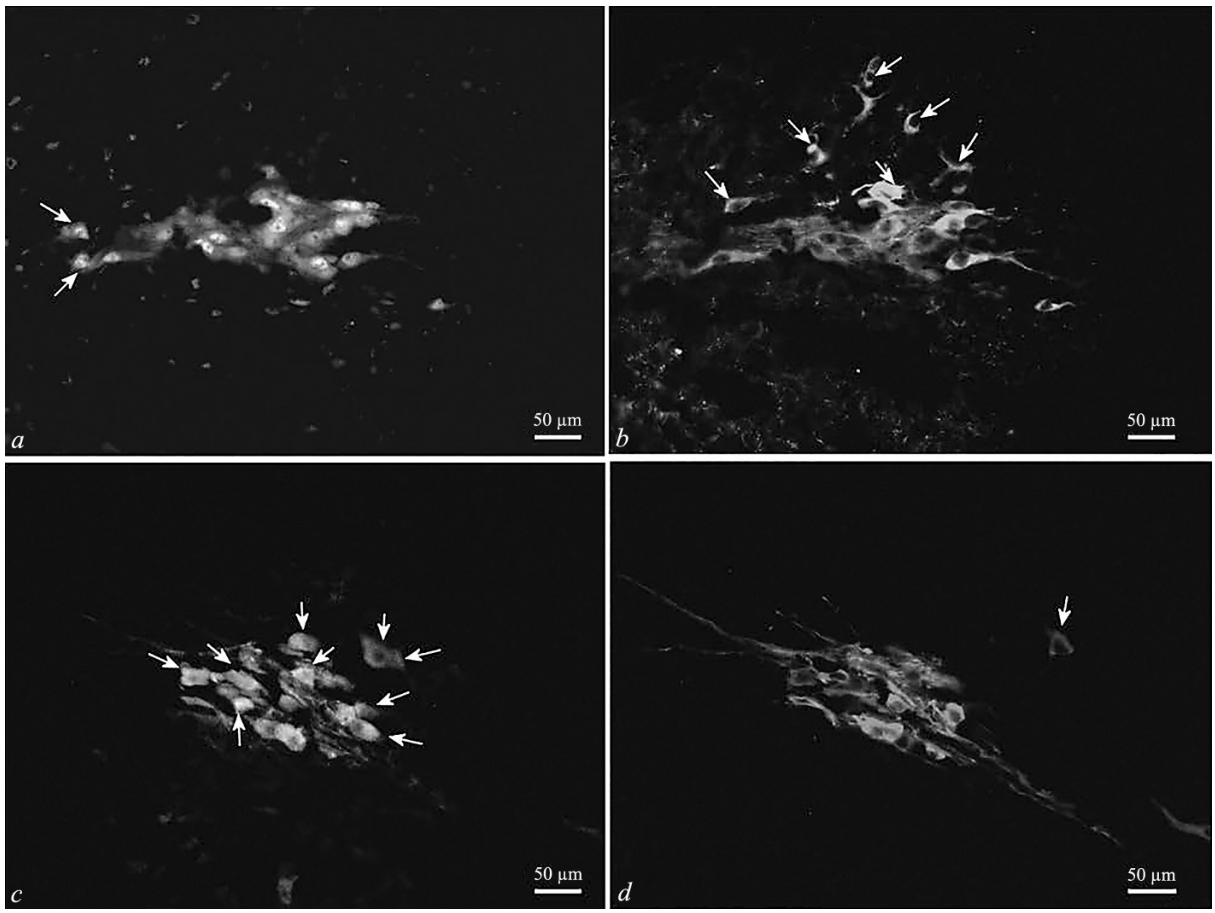

Fig. 2. Parallel reactions for CAT (*a*, *c*) and NOS (*b*, *d*) of neurons in the ILp nucleus of segment T2 of the rat spinal cord: neonatal (*a*, *b*) and 30 days old (*c*, *d*). Arrows show neurons immunopositive for only one marker (only CAT or only NOS). Fluorescence. FITC (*a*, *c*), Cy3 (*b*, *d*).

using Student's *t* test, ANOVA, and the Mann–Whitney U test. Values were regarded as significant at $p < 0.05$.

Results. Studies of the spinal cord revealed NOS in the autonomous sympathetic nuclei – ILp, ILf, IC, and ICpe – from birth in all animals (Fig. 1). Occasional NOS-positive cells were also seen in the dorsal horn, intermediate gray matter, and ventral part of the area around the central canal, which is indicated by published data not to be part of the autonomic nervous system [11]. Most NOS-containing neurons in sections were round or fusiform. Occasional triangular cells were also seen.

The largest numbers of NOS-positive sympathetic preganglionic neurons in rats of all ages were seen in the ILp nucleus (Table 1; see Fig. 1). During the first two months of life, the relative contents of NOS-positive neurons in the ILf, IC, and ICpe nuclei increased significantly, while that in the ILp nucleus decreased.

The mean profile field are of NOS-immunopositive preganglionic neurons increased during ontogeny form birth to 10 days of life, from 176 ± 8 to 208 ± 14 μm^2, after which it remained essentially unaltered. In neonatal and 10-day-old rats, most neurons in the ILp nucleus were NOS-positive and simultaneously contained the enzyme choline acetyltransferase (Fig. 2, Table 2). However, these age groups also contained a population of NOS-positive/CAT-negative neurons (26% in neonates and 8% in 10-day-old animals; these cells were not seen in older animals. During the first 20 days of life, the proportion of NOS-immunopositive neurons in the ILp nucleus decreased significantly, while the proportion of CAT-positive neurons, conversely, increased. In rat pups aged 30 days, about 45% of preganglionic sympathetic spinal cord neurons were NOS-immunonegative. This proportion remained almost unaltered thereafter.

NOS-positive neurons in the ILf nucleus were CAT-positive in all animals. The contents of NOS-positive/CAT-negative, NOS-positive/CAT-positive, and NOS-negative/CAT-positive neurons in the IC and ICpe nuclei of all animals showed no significant differences in different age groups. Thus, a developmental change in NO-ergic sympathetic transmission occurred in early postnatal ontogeny, apparent as a decrease in the number of NOS-expressing sympathetic preganglionic neurons.

Discussion. The present study showed that the majority of sympathetic preganglionic neurons in rats contain NOS from birth to old age and that NOS is colocated with the acetylcholine synthesis enzyme choline acetyltransferase, which is consistent with published data [14]. However, the ILp nucleus in rats up to day 10 of age contained a group of NOS-positive/CAT-negative neurons, which was not seen in older animals. During the first 20 days, the proportion of NOS-immunopositive neurons in this area decreased significantly, while that of CAT-positive neurons, conversely, increased. Preganglionic sympathetic fibers form synapses with neurons in the para- and prevertebral ganglia, which are NOS-negative in rodents [6, 9].

Previous studies have shown that NOS-immunoreactive neurons in the metasympathetic intramural intestinal ganglia in mice and humans mature earlier during embryogenesis than cholinergic neurons [7, 13]. In the late embryonic and early postnatal periods, NOS is expressed by a larger number of neurons than in older individuals [7].

NO has been shown to modulate synaptic function in various parts of the brain. Thus, in the basal ganglia, NO produces an almost two-fold increase in acetylcholine secretion [12]. NO also plays an important role in neuroplasticity, promoting dendrite development and synapse formation [4, 8]. Synaptic transmission in sympathetic ganglia in rats is known to reach its final form by day 20 of life [10], so it is likely that NO serves as a trophic factor, facilitating synapse maturation in early postnatal ontogeny.

Conclusions. The expression of neuronal NOS decreased and the number of CAT-containing neurons increased in preganglionic sympathetic neurons during early postnatal ontogeny. Thus, it can be suggested that NO promotes improvements in synaptic transmission in autonomic ganglia during the prenatal and early postnatal period, which at this time is still immature.

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The authors have no conflicts of interests.

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