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Changes of the Expression of Neuronal NO-Synthase in Rat Sympathetic Ganglia during Ontogeny

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Expression of neuronal NO synthase in the sympathetic cranial cervical ganglion and stellate ganglion in rats during postnatal ontogeny was studied by immunohistochemistry and Western blotting. In the sympathetic ganglia, neuronal NO synthase-immunoreactive neurons were absent in all rats. In the stellate and cranial cervical ganglia, the expression of neuronal NO synthase and the density of immunoreactive fibers increased in early postnatal ontogeny from the moment of birth to the age of 30 days and then decreased. Thus, we observed heterochroneous expression of neuronal NOS in the preganglionic somata in the spinal cord and in the preganglionic fibers in the sympathetic ganglia during ontogeny.

Key Words: *NO synthase; sympathetic ganglia; immunohistochemistry; Western blotting; ontogeny*

Nitric oxide (NO) synthesized by neuronal NO synthase (nNOS) is an intracellular and intercellular transmitter that performs various signaling functions. Most physiological effects of NO are realized through stimulation of soluble guanylate cyclase and accumulation of cGMP [5,8,12]. It has been shown that NO can modulate synaptic function and also acts as a factor promoting cell proliferation, growth of nerve fibers, and formation of synapses [8,11,12].

nNOS is detected in neurons of the central and peripheral nervous systems, including the autonomic nervous system. In rodents, nNOS is absent in the sympathetic ganglia [4,7]. However, the vast majority of mammalian preganglionic neurons contain nNOS that is co-localized with acetylcholine synthesis enzyme choline acetyltransferase (ChAT) [1,6,10].

In early postnatal ontogeny, the neurotransmitter composition of ganglionic neurons in the sympathetic ganglia, the connections of the neurons with the target organs, and synaptic transmission undergo changes [2,4,9]. In the spinal cord, the number of sympathetic preganglionic neurons expressing nNOS decreases, and the number of $ChAT^+$ neurons increases during this period [3,10]. However, changes of the expression of nNOS in the sympathetic ganglia during ontogeny remains poorly studied.

Here we studied the expression of nNOS in sympathetic ganglia during ontogeny using immunohistochemical methods and Western blotting.

MATERIALS AND METHODS

The study was carried out on 80 white Wistar female rats at the age of 1-3, 10, 20, 30 days, 1 and 2 years in compliance with the "Regulations for Animal Experiments". Cranial cervical (SCG) and stellate (SG) ganglia were used for the study.

For immunohistochemical studies, SCG and SG were fixed in 4% paraformaldehyde in 0.01 M PBS (pH 7.4), cryoprotection was performed in a 30% sucrose solution. Serial cross-sections (14 μ) of the SCG and SG were prepared on a Shandon E cryostat (Thermo Fisher Scientific). nNOS was detected using primary rabbit polyclonal anti-nNOS antibodies (dilution 1:300; Abcam) and secondary donkey anti-rabbit IgG antibodies conjugated with FITC (dilution 1:100; Jackson ImmunoResearch Laboratories) fluoresceing in the green region of the spectrum.

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The sections were washed in PBS and embedded in VectaShield immunofluorescence medium (Vector Laboratories). To exclude non-specific reaction, some sections were incubated without primary and/or secondary antibodies.

The preparations were examined under an Olympus BX43 microscope (Olympus Corporation) equipped with a set of fluorescent filters. Images were obtained using a cooled digital camera TCC-5.0ICE (Tucsen). The density of the fibers was determined using ImageJ software as the percentage of the area occupied by $nNOS^+$ fibers from the total area of the ganglion in the section.

For Western blotting, the ganglia were homogenized in a lysis buffer. Each tissue lysate was diluted in the sample buffer (Bio-Rad) and denatured at 95°C for 5 min. An equivalent volume of samples were loaded and fractionated by 10% PAAG electrophoresis and transferred to PVDF membranes (AppliChem). The membranes were blocked with a blocking solution containing 3% skimmed milk powder (AppliChem) in TBS-T (0.1% Tween-20, 0.2 mM Tris, 137 mM NaCl) for 30 min at room temperature. After washing with TBS-T, the membranes were incubated overnight at 4°C with primary antibodies (Abcam): rabbit polyclonal antibodies against nNOS (1:5000) and against GAPDH (1:2500) diluted in the same blocking solution. After washing with TBS-T, the membranes were incubated with secondary antibodies (goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000; Abcam). Immunoblots were detected by chemiluminescence (ECL Prime western blot detection reagent, Bio-Rad) with the Syngene G:BOX Chemi XR5E gel-documenting system (Syngene). Chemiluminescent signals were quantified using Gene Tools Gel Analysis software (Syngene), and their optical density was expressed relative to GAPDH. Protein molecular weight markers were included in each Western blot analysis.

Statistica 10 (StatSoft, Inc.) was used to determine arithmetic means and standard errors of the means and ANOVA analysis was used to find differences between the mean values; the differences were considered significant at $p<0.05$.

RESULTS

Immunohistochemical studies revealed no $nNOS^+$ sympathetic neurons in the SCG and SG in all animals. Some weakly expressed bundles of $nNOS^+$ sympathetic fibers were detected in newborns. The density of $nNOS^+$ fibers significantly increased during the first month of life ($p<0.05$; Table 1). Intensive fluorescence of $nNOS^+$ fibers was observed in 20-day-old animals, reaching a maximum in 30-day-old animals. In old animals, the intensity of fluorescence and fibers density decreased in comparison with those in 30-day- and 1-year-old rats ($p<0.05$). There were no significant differences between the density of $nNOS^+$ fibers in the SCG and SG.

The expression of nNOS in the SCG and SG was determined by the method of Western blotting. In newborns, bands corresponding to 150-kDa protein were detected in both ganglia (Table 1). Protein expression significantly increases in the early postnatal ontogeny from birth to 30 days of life and decreases in old animals. At the same time, no significant differences in the expression of nNOS between the SCG and SG were found.

Thus, the results of this study indicate the absence of $nNOS^+$ neurons in the rat sympathetic ganglia and an increase in the density of $nNOS^+$ fibers during the first 30 days of life. This confirms the data of previous studies of NADPH-diaphorase in the sympathetic ganglia. However, in the spinal cord of newborns and 10-day-old rats, the majority of sympathetic preganglionic neurons contained nNOS and ChAT. In contrast to sympathetic ganglia, the relative content of $nNOS^+$

TABLE 1. Changes of nNOS expression in SCG and SG (*M*±*SE*)

Age	Density of $nNOS^+$ fibers, % of ganglion cross-section area		Expression of $nNOS^+$-fibers, % relative to GAPDH	
	SCG	SG	SCG	SG
Newborn	0.20±0.02*,+	0.20±0.03*	0.46±0.04*,+	0.48±0.03*,+
10 days	0.50±0.12+	0.40±0.09	0.61±0.03+	0.66±0.03**
20 days	8.00±0.81*	9.00±1.32*	0.83±0.09*	0.81±0.05*
30 days	12.00±1.53*,+	13.00±2.52*	0.94±0.06*,+	0.94±0.05*,+
1 year	12.00±0.68*,+	13.00±1.82*	0.92±0.05*,+	0.91±0.04*,+
2 years	7.00±0.57*	8.00±0.76*	0.82±0.03*	0.81±0.04*

Note. $p<0.05$ in comparison with *10-day-old animals, +2-year-old animals.

neurons in the spinal cord significantly decreased, while the content of $ChAT^+$ neurons increased during the first 20 days of life [10].

NO plays an important role in neuroplasticity, promotes the growth of dendrites and formation of synapses [8,11,12]. It is known that the formation of synaptic transmission in sympathetic nodes is completed by day 20 of life [9]; therefore, NO can serve as a trophic factor contributing to maturation of synapses in the early postnatal ontogeny.

Thus, there is a heterochrony of the expression of nNOS in the bodies of preganglionic neurons in the spinal cord and in the preganglionic fibers in the sympathetic ganglia. In sympathetic ganglia, the expression of nNOS and the density of $nNOS^+$ sympathetic preganglionic fibers increased in early postnatal ontogeny from birth to 30 days of life and decreased in old animals. In contrast, in the spinal cord, the percentage of $nNOS^+$ sympathetic preganglionic neuronal somata decreased during the first 20 days of life.

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