ISSN 2079-0570, Advances in Gerontology, 2019, Vol. 9, No. 2, pp. 248–253. © Pleiades Publishing, Ltd., 2019.
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Age-Related Changes in Sympathetic Innervation of the Stomach in Rats

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Received September 13, 2018; revised October 8, 2018; accepted October 16, 2018

Abstract—Sympathetic innervation of the stomach is carried out by the prevertebral ganglia of the solar plexus. The localization and neurochemical composition of neurons innervating the stomach in the postnatal ontogenesis of rats have been studied by the method of retrograde axon transport of Fast Blue. In all animals, the celiac ganglia had more labeled neurons than the superior mesenteric ganglion. The number of labeled neurons increased in the first ten days of life and then did not change until senescence. All labeled neurons innervating the stomach contain tyrosine hydroxylase, the catecholamine synthesis enzyme. The proportion of labeled neuropeptide Y-immunopositive neurons did not change during ontogeny; the percentage of labeled calbindin-immunoreactive neurons decreased in the first month of life.

Keywords: ontogenesis, sympathetic innervation, stomach, neuropeptide Y, calbindin
DOI: 10.1134/S2079057019020097

INTRODUCTION

The prevertebral ganglia are located far from CNS. The two paired celiac ganglia (CG) and unpaired superior mesenteric ganglion (SMG) of the solar plexus mostly contribute to the sympathetic innervation of abdominal organs, including the gastrointestinal tract (GIT). The neurons innervating the upper GIT regions are situated in the rostral CG, while the caudal neurons are mostly responsible for control over the distal parts of the intestine [19].

About 90% of neurons in the sympathetic para- and prevertebral ganglia are catecholaminergic and contain tyrosine hydroxylase (TH), the enzyme of catecholamine synthesis [4, 5, 17]. Most TH-positive neurons of the sympathetic ganglia are also neuropeptide Y-positive (NPY) [4–6]. Around one third of the neurons in the paravertebral ganglia contain calbindin (CA), the calcium-binding protein [2–5, 10]. The percentage of CA-immunoreactive neurons is significantly lower in the CG [2, 5]. Unlike in large mammals, no synthesis of nitrogen oxide is found in sympathetic ganglia of rodents [8, 13].

The mediator composition of sympathetic neurons changes during ontogeny, along with functional maturation, and these changes may occur under the influence of numerous trophic factors [5]. Catecholamines and NPY are detected in sympathetic ganglia on embryonic stage [5, 6]. The amount of NPY-containing sympathetic neurons increases in postnatal ontogeny from the moment of birth until the end of the second month of life, while the number of CA-containing neurons simultaneously decreases [2, 15, 16].

It was also found that the number of connections between the neuronal ganglia and target organs changes in paravertebral ganglia [1, 9, 16]. Nevertheless, there are extremely few data on age-related modifications of GIT innervation from the paravertebral ganglia in the contemporary literature. Thus, the purpose of this study was to determine the neurochemical characteristics of stomach innervation in rats in the process of lifespan development from the moment of birth to senescence.

MATERIALS AND METHODS

The study was conducted on 35 Wistar male rats at the age of 1, 10, and 20 days; 1, 2, and 6 months; and 3 years from birth (five animals in each age group).

Fast Blue label (FB) (Polysciences, United States) was administered under the serous layer of the stomach with a microsyringe (2% solution in phosphate buffer saline (PBS), 0.01 M, pH 7.4) at a dose of 10 μL in each injection. The postoperative period lasted 24 h for infant rats younger than 1 month, inclusively, and

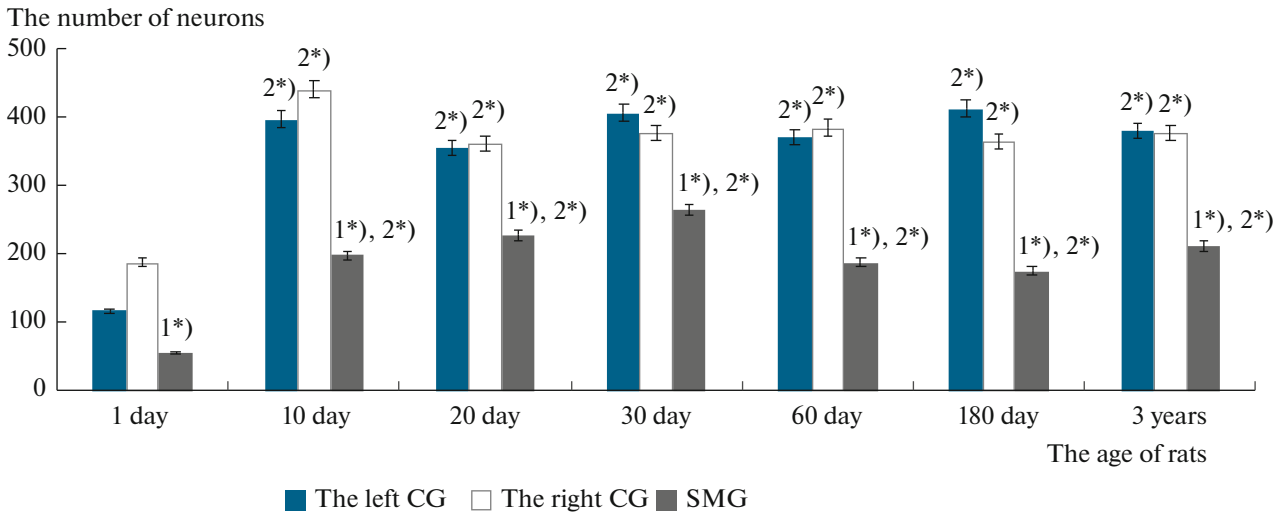

Fig. 1. Number of neurons in the prevertebral sympathetic ganglia innervating the stomach in rats of different ages. 1*) $p < 0.01$, the differences are significant as compared to the left and right CG; 2*) $p < 0.05$, the differences are significant as compared to newborn animals.

48 h for 2-month-old, 6-month-old, and 3-year-old animals.

After the injection of a lethal dose of urethane (3 g/kg intraperitoneal), the animals were transcardially perfused with sodium solution of heparin and afterwards with a fixative mixture of 4% paraformaldehyde in PBS. The CG and SMG were examined. The ganglia were fixated in the same mixture for 2 h, washed three times in PBS for 30 min, and left in 20% saccharose solution (pH 7.4) for one night. Sections of fixated material 14 μm in thickness were prepared with a thermostatic cooler.

To indicate TH-, NPY-, and CA-containing neurons, the method of labeled antibodies was applied according to the scheme described in our previous works [2, 4]. We used Abcam (United States) primary antibodies of sheep against TH (1 : 1000 dilution) and of rabbit against NPY and CA (1 : 500 dilution). Secondary antibodies were conjugated with fluorescein isothiocyanate fluorochrome (FITC, Jackson Immunosearch, United States) diluted as 1 : 100 and given a green fluorescence.

Sections were preincubated for 30 min at room temperature in PBS with the addition of 10% serum, 1% Triton X-100, 0.1% bovine serum albumin, and 0.05% thimerosol. The sections were then incubated with primary antibodies for 24 h at room temperature. After quick washing in PBS, the sections were incubated with secondary antibodies for 2 h. The sections were further washed in PBS and captured in medium for immunofluorescence (VectaShield, Vector Laboratories, United States).

The samples were analyzed with an Olympus BX43 fluorescent microscope (Tokio, Japan) with the corresponding color filter set and a coolable CCD digital camera Tuscen TCC 6.1ICE and ISCapture 3.6 software (China). FB-containing neurons were detected by blue fluorescence and immunopositive neurons were green. Image J software (NIH, United States, http://rsb.info.nih.gov/ij/) was used to analyze the percentage of immunopositive neurons on digital images of histological specimens.

Statistical analysis of the results was conducted with Sigma Plot (StatSoft, United States) software. All values are presented as mean ± standard error ($M \pm m$). The significance of the differences between the mean values was determined by one-way ANOVA with Bonferroni correction. The differences were statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The labeled neurons were identified in rats of all age groups after FB injection in the stomach wall. In the process of lifespan development, the number of neurons innervating the stomach significantly increased ($p < 0.01$) in all observed ganglia of solar plexus over the first ten days of life and remained unchanged also in elderly animals ($p > 0.05$, Fig. 1). The majority of neurons were statistically detected in the CG in all age groups, in contrast with the SMG ($p < 0.01$).

All FB-containing neurons of the solar plexus were TH-immunopositive from the moment of birth. No statistical differences were found between the mean sectional area of the FB-containing and overall population of TH-immunoreactive neurons in the left and right CG and SMG ($p > 0.05$). However, the labeled neurons of the left and right CG had a statistically wider area in comparison with the SMG in ten-day-old and older rats (Table 1).

Some of the labeled neurons in the CG and SMG contained NPY and CA (Fig. 2–4). The percentage of NPY-immunopositive neurons innervating the stomach varied from 40 to 50%, even in newborn rats (Fig. 2, 3), and remained unchanged ($p > 0.05$) in the aging process. Most of the neurons of newborn (48 ± 3.2%) and ten-day-old (44 ± 3.7%) rats contained CA (Fig. 3, 4), although the rate of CA-immunoreactive neurons decreased statistically, amounting to several percentage points from the 30th day.

From the 20th day of life, the mean sectional area of NPY-immunopositive neurons containing FB was smaller in the SMG than in the CG ($p > 0.05$, Table 2). Unlike TH- and NPY-, CA-containing labeled neurons of the CG and SMG did not vary significantly in the mean sectional area ($p > 0.05$, Table 3).

Therefore, the results suggest that the connections between the sympathetic neurons of the solar plexus and stomach can be found even in newborn animals. Most of the neurons innervating the stomach are located in the CG, in contrast with the SMG. Over the first ten days of life, the number of neurons innervating the stomach increases and remains stable throughout the life of animals, including elderly subjects. According to the literature data, the percentage of neurons innervating thoracic organs and cervical vessels also rises over the first ten days of life [1, 9].

Elderly rats did not show statistical differences in the number of sympathetic neurons innervating the stomach or in their neurochemical characteristics when compared to younger mature animals. Nevertheless, age-related involution of the sympathetic innervation of inner organs is typical for humans and is less prominent in rats [5]. The demonstrated differences should be considered during the animal-to-human extrapolation of age-related experimental data.

All labeled neurons innervating the stomach contained TH, the enzyme of catecholamine synthesis; herewith, some of them are NPY- and CA-immunopositive. The rate of NPY-immunopositive neurons did not change during ontogeny, and the percentage of CA-immunoreactive neurons diminishes over the first month of life. Earlier experiments demonstrated that the rate of NPY-immunopositive sympathetic ganglial neurons innervating the heart and vessels of the skeletal muscles remained constant over ontogeny from the moment of birth [15, 16]. Our data on the percentage of TH- and NPY-immunopositive neurons innervating stomach in rats correspond with the results shown on stomach innervation in pigs [18, 21]. A reduction of the amount of CA-containing sympathetic ganglial neurons was demonstrated for the early ontogeny of different animal species in our previous studies [2–4, 14].

Age-related changes in the CA composition may be associated with its impact on the antiapoptotic defense of cell [7] and the protective function in calcium-dependent neurotoxicity [12]. Of all the factors regulating synaptic development and plasticity, CA is essential in the maintenance of a particular concentration of calcium ions that may vary in space and time [11, 20].

Table 1. Mean values of the sectional area of TH-immunoreactive neurons innervating the stomach in the ganglia of the solar plexus in rats of different ages (200 neurons from each age group), μm^2

Age	Left CG	Right CG	SMG
Newborn	184 ± 15	183 ± 18	191 ± 11
10 days	297 ± 23	301 ± 25	232 ± 22*
20 days	415 ± 33	435 ± 36	316 ± 38*
30 days	502 ± 28	515 ± 33	451 ± 31
60 days	634 ± 34	641 ± 29	545 ± 37*
180 days	709 ± 42	713 ± 44	612 ± 44
3 years	741 ± 51	713 ± 43	621 ± 36*

Here and in Table 2 *—$p < 0.05$, the differences are significant as compared to the left and right CG.

Table 2. Mean values of the sectional area of NPY-immunoreactive neurons innervating the stomach in the ganglia of the solar plexus in rats of different ages (200 neurons from each age group), μm^2

Age	Left CG	Right CG	SMG
Newborn	171 ± 6	175 ± 9	180 ± 11
10 days	284 ± 14	262 ± 18	246 ± 28
20 days	453 ± 13	463 ± 23	355 ± 26*
30 days	557 ± 22	544 ± 27	436 ± 31*
60 days	648 ± 34	661 ± 38	541 ± 32*
180 days	722 ± 35	718 ± 46	583 ± 37*
3 years	734 ± 37	721 ± 38	612 ± 35*

Table 3. Mean values of the sectional area of CA-immunoreactive neurons innervating the stomach in the ganglia of the solar plexus in rats of different ages (200 neurons from each age group), μm^2

Age	Left CG	Right CG	SMG
Newborn	168 ± 7	173 ± 7	175 ± 8
10 days	346 ± 18	351 ± 17	347 ± 16
20 days	433 ± 25	418 ± 26	421 ± 22
30 days	526 ± 24	517 ± 22	509 ± 18
60 days	595 ± 23	591 ± 28	604 ± 31
180 days	689 ± 27	704 ± 33	693 ± 32
3 years	692 ± 25	704 ± 29	710 ± 38

CONCLUSIONS

The results of this study suggest that the connections between the neurons of sympathetic ganglia and

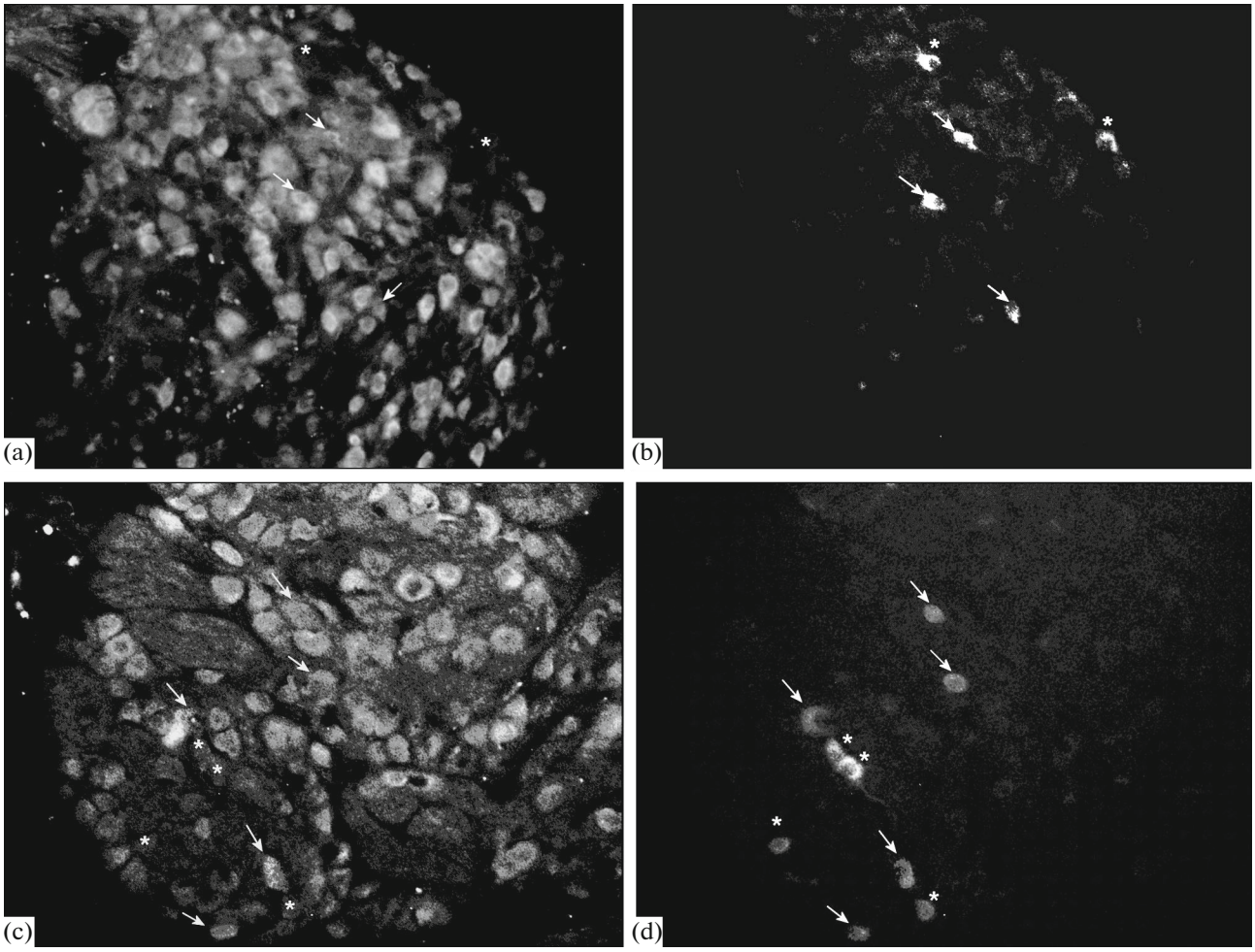

Fig. 2. Photomicrograph of NPY-immunoreactive (a, c) and FB-containing (b, d) neurons in the left CG in rats of different ages; (a, b) of 10-day-old; (c, d) of 30-day-old infant rat. Arrows indicate double-labeled neurons (NPY and FB); asterisks show NPY-negative labeled neurons; FITC fluorescence is marked in green and FB in blue; object lens 20, ocular 10.

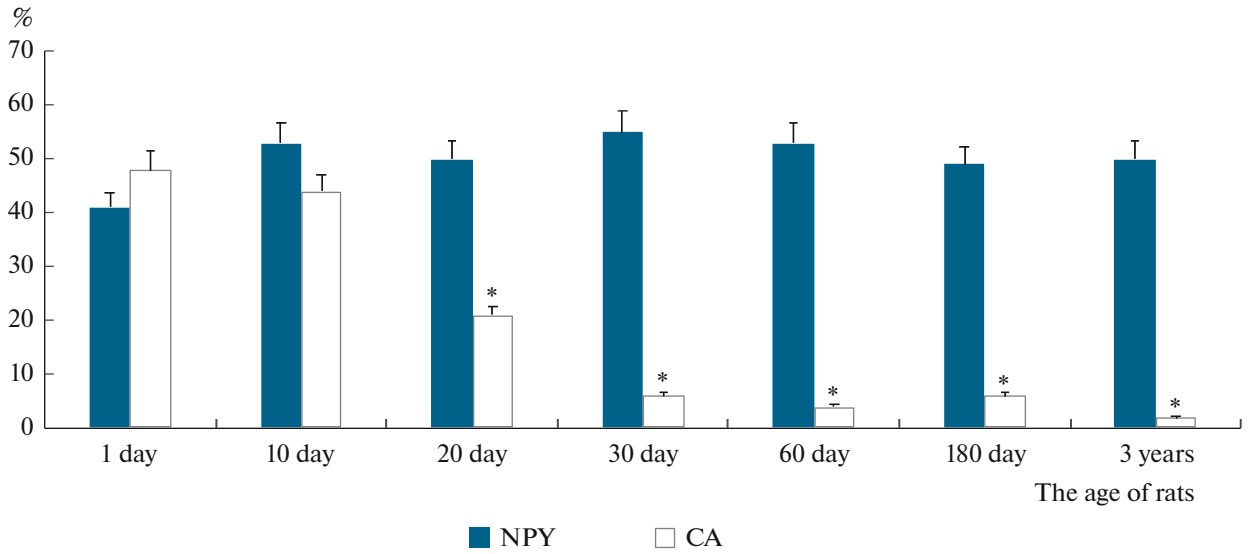

Fig. 3. Percentage of NPY- and CA-immunopositive neurons innervating the stomach in rats during postnatal ontogeny. * $p < 0.001$, the differences are significant in comparison with 10-day-old animals.

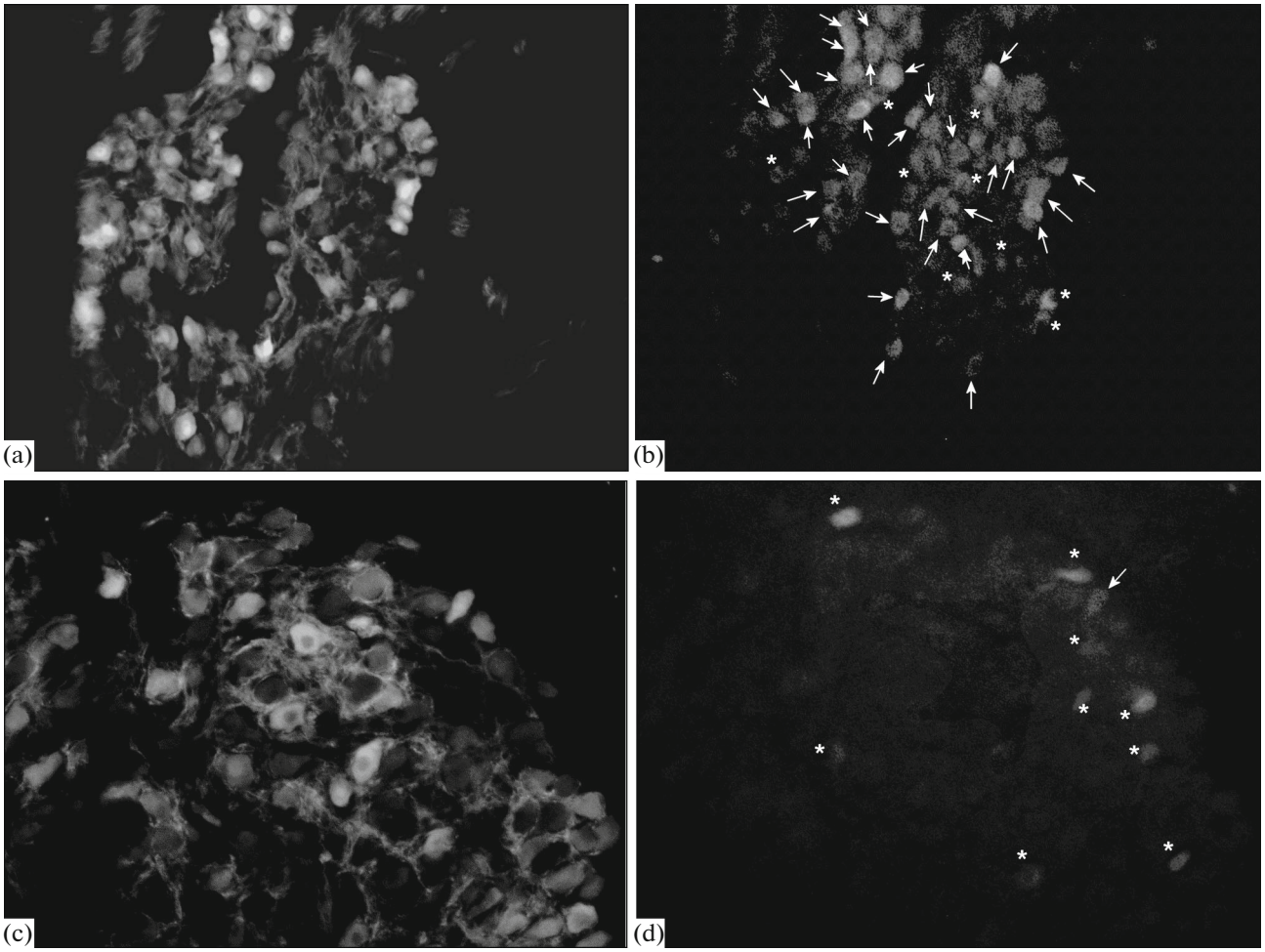

Fig. 4. Photomicrograph of CA-immunoreactive (a, c) and FB-containing (b, d) neurons in the left CG in rats of different ages; (a, b) of 10-day-old; (c, d) of 30-day-old infant rat. Arrows indicate double-labeled neurons (NPY and FB); asterisks show NPY-negative labeled neurons; FITC fluorescence is marked in green; FB; object lens 20, ocular 10.

target organs are formed in rats already from the moment of birth. The majority of neurons innervating the stomach are located in celiac ganglia compared to superior mesenteric ganglion. All labeled neurons innervating the stomach contain tyrosine hydroxylase, the enzyme of catecholamine synthesis; moreover, some of them re NPY- and CA-immunopositive. The percentage of NPY-immunopositive neurons remains unchanged during ontogeny, while the amount of CA-immunoreactive neurons decreases over the first month of life.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. The animals were kept, sacrificed, and tested in accordance with the Rules of Working with Experimental Animals (order of the Ministry of Health of the Russian Federation no. 775, August 12, 1997).

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Translated by E. Sherstyuk