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Age-dependent patterns of somatostatinergic neurons in sympathetic paravertebral ganglia

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Abstract

Aim. We aimed to determine the content of neurons expressing somatostatin (SST) and their colocalization with cells expressing tyrosine hydroxylase (TH) and neuropeptide Y (NPY) in the cranial cervical ganglion (CCG) and celiac plexus in rats.

Materials and methods. We used 30 white male Wistar rats of six age groups (5 rats per group): newborn pups, 10-, 20-, 30-, and 60-day-old pups, and 24-month-old pups. We incubated their ganglia sections with primary antibodies against SST, NPY, and TH, as well as with secondary antibodies conjugated with fluorochromes. We evaluated the ratio between immunoreactive (IR) neurons with a visible nucleolus and excessive fluorescence and the total number of neurons, as well as the average cross-sectional area, by ImageJ software (NIH, USA).

Results. SST-IR neurons were not found in the CCG. However, the immunoreaction (as granules) was revealed in most perikaryons at the celiac plexus for SST and NPY with a rather homogeneous distribution for TH. The ratio of ST-IR neurons reached 33% in pups, doubled during the first month of life, and then remained constant (70–73%). No statistically significant differences were found between the ratios of SST-IR neurons of the cranial mesenteric ganglion (CMG) and celiac ganglion (CG) for all age groups. From the moment of birth to 60 days of life, the average cross-sectional area of SST-IR neurons in the CG and CMG increased by 3.4–3.9 times and then did not change until 24 months. From the 20th day of life, the average cross-sectional area of SST-IR neurons in the CG was significantly higher than that in the CMG. All SST-IR neurons in all age groups expressed TH, while 90–94% of neurons expressed NPY.

Conclusions. The content of ST-IR neurons in different sympathetic nodes is not the same: they are absent in the CCG, and their ratio and area in the celiac plexus increase during early postnatal development. This may be due to the peculiarities of innervated target organs.

Keywords: somatostatin; colocalization of somatostatin; tyrosine hydroxylase; neuropeptide Y; gastrointestinal tract; motility

MeSH terms:
GANGLIA, SYMPATHETIC – METABOLISM
GANGLIA, SYMPATHETIC – GROWTH & DEVELOPMENT
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Возрастное развитие соматостатинергических нейронов симпатических превертебральных узлов

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Аннотация

Цель. Определить содержание нейронов, экспрессирующих соматостатин (SST), и его колокализацию с тирозингидроксилазой (TH) и нейропептидом Y (NPY) в краниальном шейном ганглии (КШГ) и солнечном сплетении крысы.

Материалы и методы. Работа выполнена на 30 белых крысах-самцах линии Wistar шести возрастных групп (по 5 крыс в каждой): новорожденные, 10-, 20-, 30-, 60-суточные, 24-месячные. Срезы ганглиев инкубировали с первичными антителами к SST, NPY, TH и вторичными, конъюгированными с флуорохромами. Определяли долю иммунореактивных (IR) нейронов с видимым ядрышком и с флуоресценцией, превышающей фоновое свечение, по отношению к общему числу нейронов, а также среднюю площадь сечения с помощью программы Image J (NIH, США).

Результаты. SST-IR нейроны отсутствовали в КШГ. Иммунореактивный материал обнаруживался в области тел большинства нейронов солнечного сплетения в виде гранул для SST и NPY и располагался относительно гомогенно для TH. Доля SST-IR нейронов составляла 33% у новорожденных, увеличивалась в два раза в первый месяц жизни и далее оставалась постоянной (70–73%). Не установлено статистически значимых различий по долям SST-IR нейронов между краниальным брыжеечным ганглием (КБГ) и чревным ганглием (ЧГ) во всех возрастных группах. С момента рождения и до 60 суток жизни средняя площадь сечения SST-IR нейронов в ЧГ и КБГ увеличивалась в 3,4–3,9 раза и далее не менялась до 24 мес. С 20-х суток жизни средняя площадь сечения SST-IR нейронов в ЧГ была статистически значимо выше, чем в КБГ. Все SST-IR нейроны во всех возрастных группах содержали TH и 90–94% нейронов колокализовали NPY.

Заключение. Содержание SST-IR нейронов в различных симпатических узлах гетерохронно: они отсутствуют в КШГ, а их доля и площадь в солнечном сплетении в раннем постнатальном онтогенезе увеличивается. Это может быть связано с особенностями иннервируемых органов-мишеней.

Ключевые слова: соматостатин; колокализация соматостатина; тирозингидроксилаза; нейропептид Y; желудочно-кишечный тракт; моторика
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Abbreviations:

CY3 – indocarbocyanine
FITC – fluorescein isothiocyanate
IR – immunoreactive
NPY – neuropeptide Y
SST – somatostatin
SSTR – somatostatin receptor
TH – tyrosine hydroxylase
CMG – cranial mesenteric ganglion
CCG – cranial cervical ganglion
CG – celiac ganglion

Somatostatin (SST) is a polypeptide that is a hormone and neuropeptide and may exist in one of two biologically active forms, SST-14 and SST-28, with different sizes of amino acid chains [1]. Physiological functions are mediated by 5 types of somatostatin receptors: SSTR1-SSTR5 (somatostatin receptor), which have similar structures and signal transmission mechanisms but various cellular and subcellular localizations and modes of regulation [1, 2].

SST is primarily considered a strong inhibitory factor in the endocrine system and gastrointestinal tract. The molecule manages cell division, the ability of visceral smooth muscles to contract, and the metabolism of many nutrients. As a neuropeptide, SST directly acts in the central nervous system, regulating the transmission of nerve impulses [1, 3]. SST is also a trophic factor during the neuronal development of neurons at the embryonic stage [4]. Recent studies have revealed the presence of SST in the mammalian autonomic nervous system, particularly in some sympathetic node neurons [5]. The largest proportion of these neurons produce mediators such as norepinephrine, and enzymes such as tyrosine hydroxylase (TH) are required for this synthesis. Two-thirds of the neurons contain neuropeptide Y (NPY) [6, 7].

The neurotransmitter composition of sympathetic neurons varies during the lifetime, and the neuronal localization in ganglia differs [8–10]. For instance, transient expression of SST is observed in most sympathetic ganglion neurons in rats on the 16th day of embryonic development [10, 11].

During the embryonic development of guinea pigs, SST-immunoreactive (IR) neurons in the celiac plexus may be found later than NPY-IR neurons, and the majority (75%) of SST-IR neurons continue to express NPY in later embryonic stages, but it is rarely observed in the neonatal period (<2%) [8].

After birth, a small number of SST-IR neurons are present in the prevertebral nodes in mice [13]. However, in guinea pigs, the number of SST-IR neurons in the prevertebral nodes of the celiac plexus reaches 25% and 12–15% in pigs, in contrast to their paravertebral nodes [14, 15]. Thus, in rats, the paravertebral cervicothoracic ganglion has the largest number of SST neurons in newborn pups (7% of the total number of neurons). Later, the number of SST-IR neurons decreases to 4% and remains constant after 10 days of life [9].

In the literature, we found no data about postnatal changes in neurotransmitter production for other sympathetic nodes, specifically for paravertebral cranial cervical ganglia (CCG), as well as for the celiac plexus, including prevertebral celiac ganglia (CG) and cranial mesenteric ganglia (CMG).

The study aimed to determine the postnatal content of SST-IR neurons in the CCG and celiac plexus of rats, as well as the extent of colocalization between somatostatin and other neurotransmitters, including enzymes for catecholamine synthesis (TH) and NPY.

MATERIALS AND METHODS

We used 30 white Wistar male rats of six age groups (5 rats per group): newborn pups, 10-, 20-, 30-, and 60-day-old pups, and 24-month-old pups.

Animals were killed by injecting a lethal dose of urethane (3 g/kg intraperitoneally). Then, we performed transcardial perfusion of standard phosphate buffered saline (PBS) 0.01 M, pH 7.4 (BioloT LLC, Russia) at 20–500 ml depending on age, and then with the same volume of a fixing mixture of 4% paraformaldehyde solution (Sigma, USA) in PBS. Then, we removed the CCG and ganglia of the celiac plexus (right and left CG and CMG). We held them in the same fixing mixture at room temperature for 2 hours. Furthermore, we washed the ganglia three times in PBS for 10 minutes at 5-minute intervals. The tissues were incubated in 30% sucrose solution with PBS overnight at a temperature of 4 °C. We then cut a series of 12 μm tissue sections with a cryostat.

During the next stage, we provided preconditioning for 30 minutes at room temperature in PBS with 10% donkey serum (Jackson ImmunoResearch, USA), 1% Triton X-100, 0.1% bovine serum albumin, and 0.05% thiomersal. Then, we incubated the slides with primary antibodies for 24 hours at room temperature: goat anti-SST, 1:200 (Santa Cruz, sc-7819), sheep anti-TH, 1:1000 (Abcam, ab113), and rabbit anti-NPY, 1:500 (Abcam, ab30914). After a short-term PBS wash, we proceeded with secondary antibodies for 2 hours. Secondary antibodies were conjugated with fluorochromes: fluorescein isothiocyanate (FITC), green, and indocarbocyanine (CY3), red (1:150, Jackson ImmunoResearch, USA).

The total neuronal population was stained with green fluorescent dye NeuroTrace™ Green Fluorescent Nissl Stains (Molecular Probes, USA), 1:200. Then, we washed the slides with PBS and covered them with mounting medium for fluorescence microscopy with VectaShield (Vector Laboratories, USA). Some sections were incubated with no primary and/or secondary antibodies to avoid nonspecific binding.

We examined the slides with a software-hardware complex: an Olympus BX43 fluorescence microscope (Olympus, Japan) with a set of filters (UFBWA mirror module – blue, BP460-495 excitation filter, BA510-550 barrier filter – green fluorescence; U-FGWA mirror module – green excitation, BP530-550 excitation filter, BA575-625 barrier filter – red fluorescence), a cooling SSD camera Tucsen FL-20 (Xintu Photonics, China), and a computer with an Intel Core i7 processor (Intel, USA). Images were taken and processed with Mosaic software, version 2.1 (Xintu Photonics, China).

We combined the slide images obtained by multichannel fluorescence with different fluorochrome spectra using Paint Shop Pro 7.02 software (Jasc Software, Inc., USA). We superimposed images from the same fields of view. Such a combined image displayed green, red, and overlapping as a yellow-green gradient.

We used every third section from the serial slices to identify labelled neurons. The number of labelled neurons was determined on images of slides with an area of 0.14 mm^2 obtained with ob. 20x/0.50. We counted the content of SST-IR neurons as their ratio to the total number of neurons detected using NeuroTrace Fluorescent NisslStains (given as 100%). We only examined neurons with cut nuclei and nucleoli and evaluated the levels of excessive fluorescence. We obtained the number of labelled neurons and the average cross-sectional area of SST-IR neurons with ImageJ software (NIH, USA). A manual limiting of membranes for IR cells allowed us to determine the average cross-sectional area with an increase of 200x. In total, we analysed 200 neurons in each ganglion of each age group.

Statistical processing

We assessed the normality of distributions using the Shapiro–Wilk test. The significance of the difference between the mean values was determined using a single-factor analysis of variance (ANOVA). We employed a t-test for paired comparisons, and Bonferroni correction was used for multiple comparisons. The differences were considered significant at $p < 0.05$. SigmaPlot software (Systat Software, USA) was used for statistical data analysis.

RESULTS

The results showed that SST and NPY immunolabelling were visualized as granules in the cytoplasm, while the immunoreactivity to TH remained mostly homogeneous. In most neurons, immunoreactive material was found in perikaryons. However, some cells were also labelled at proximal portions of dendrites.

The distribution of labelled neurons on slides was mostly homogeneous. We found no quantitative difference between the left and right CG. Therefore, the averaged data for both nodes are given later.

SST-IR neurons were not detected in the CCG but were numerous in the celiac plexus. Some neurons remained SST-IR in both the CG and CCG from birth to 24 months (Fig. 1).

In newborn pups, one-third of the neurons in the CG and CMG exhibited IR. The content of SST-IR neurons doubled in ontogenesis in both nodes during the first 30 days of life but remained at 70–73% until 24 months (Table 1). We observed no significant difference between the content of SST-IR from the CMG and CG for all age groups.

The average cross-sectional area of SST-IR neurons increased in the CG and CMG from the moment of birth

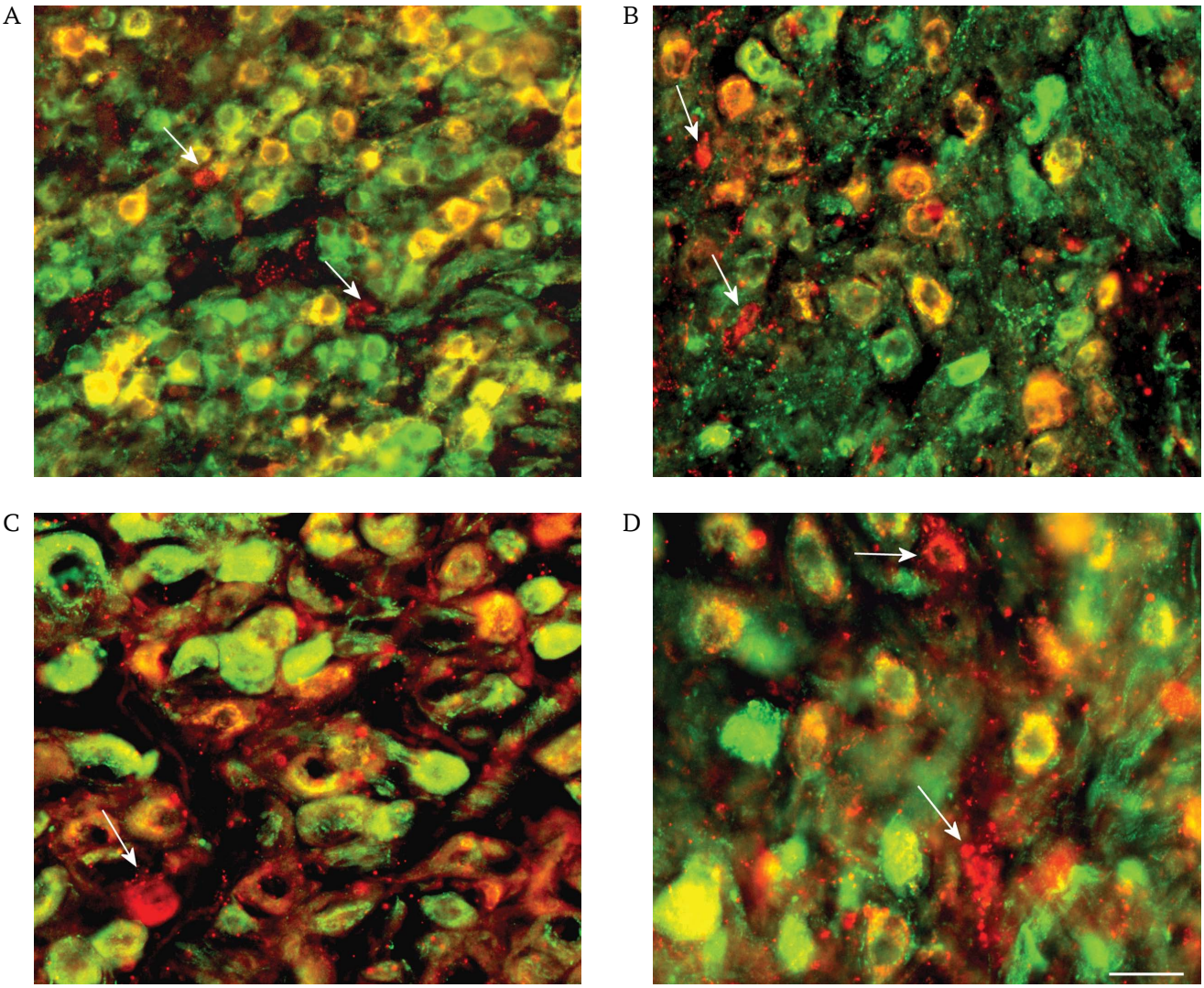

FIG. 1. Content of neurons expressing somatostatin (red) and neuropeptide Y (green) and their colocalization (yellow) in celiac ganglia of rats at different ages.
Microphotograph: ob. 20, oc. 10. Scale: 30 μm.
Somatostatinergic neurons immunonegative for neuropeptide Y are indicated by arrows. Fluorescence: fluorescein isothiocyanate and indocarbocyanine.
Age of the rat: A – newborn pup, B – 10-day-old, C – 60-day-old, D – 24-month-old.
РИС. 1. Содержание в чревном ганглии у крыс разных возрастов нейронов, экспрессирующих соматостатин (красные) и нейропептид Y (зеленые), и их колокализация (желтый цвет).
Микрофото: об. 20, ок. 10. Масштаб: 30 мкм.
Иммунонегативные к нейропептиду Y соматостатинергические нейроны указаны стрелкой. Флуоресценция: флуоресцеин-изотиоцианат и индокарбоцианин.
Возраст крысы: A – новорожденная, B – 10-суточная, C – 60-суточная, D – 24-месячная.

up to 60 days of life (Table 2). From the 20th day of life, the average cross-sectional area of SST-IR neurons in the CG was significantly higher than that observed in the CMG in rats.

In all age groups, from birth up to 24 months, all SST-IR neurons in the CG and CMG expressed the enzyme of catecholamine synthesis (TH). At the same time, regardless of age, the largest proportion of SST-IR neurons (90–94%) contained NPY (Figs. 1, 2). No significant difference was found between the content of SST-IR neurons with NPY in different age groups (Fig. 2).

DISCUSSION

The results of this study demonstrate that SST-IR neurons are present in the prevertebral CG and CMG at the moment of birth, while no SST-IR neurons are found in the paravertebral CCG at the same time point. In rats, the content of SST-IR neurons in the prevertebral nodes exceeds the parameter values for other mammals, including mice, guinea pigs, pigs, and humans. In mice and humans, some solitary SST-IR neurons are found. In pigs and guinea pigs, the number of SST-IR neurons in prevertebral nodes reaches 12–15% and 25%, respectively [5, 6, 13, 14].

Table 1. **The content of somatostatinergic neurons in postnatal sympathetic ganglia of rats**
Таблица 1. **Доля соматостатинергических нейронов в симпатических узлах крыс в постнатальном онтогенезе**

Age / Возраст	The content of SST-IR neurons, % / Доля SST-IR нейронов, %		*p* value / Значение *p*
	Celiac ganglion / Чревный ганглий	Cranial mesenteric ganglion / Краниальный брыжеечный ганглий	
Newborn pup / Новорожденный	34 ± 3.1[a]	33 ± 3.3[a]	n.s.
10-day-old / 10 суток	42 ± 2.7[a]	45 ± 4.1[a]	n.s.
20-day-old / 20 суток	55 ± 4.9[a]	53 ± 5.8[a]	n.s.
30-day-old / 30 суток	70 ± 6.2	72 ± 7.9	n.s.
60-day-old / 60 суток	73 ± 5.9	73 ± 6.2	n.s.
24-month-old / 24 месяца	71 ± 5.6	71 ± 7.1	n.s.

Note: the content of somatostatinergic neurons is given as a percentage of the total number of neurons. Data for celiac ganglia are presented as average values for right and left nodes.
[a] $p < 0,05$ compared to 60-day-old.
SST-IR – somatostatin immunoreactive; n.s. – not significant.
Примечание: доля соматостатинергических нейронов представлена в процентах от общего числа нейронов. Данные по чревному ганглию представлены усредненными значениями по правому и левому узлам.
[a] $p < 0,05$ по сравнению с 60-суточным.
SST-IR – somatostatin immunoreactive, соматостатин иммунореактивные; n.s. – not significant (не значимо).

Table 2. **Cross-sectional area of somatostatinergic neurons in the sympathetic ganglions of rats at different ages**
Таблица 2. **Площадь поперечного сечения соматостатинергических нейронов в симпатических узлах крыс разных возрастов**

Age / Возраст	Cross-sectional area of SST-IR neurons, μm² / Площадь поперечного сечения SST-IR нейронов, мкм²		*p* value / Значение *p*
	Celiac ganglion / Чревный ганглий	Cranial mesenteric ganglion / Краниальный брыжеечный ганглий	
Newborn pup / Новорожденный	172 ± 7,2[a]	167 ± 8,1[a]	n.s.
10-day-old / 10 суток	272 ± 10,7[a]	273 ± 12,6[a]	n.s.
20-day-old / 20 суток	453 ± 11,2[a]	382 ± 15,5[a]	<0,05
30-day-old / 30 суток	574 ± 22,2[a]	485 ± 19,8[a]	<0,05
60-day-old / 60 суток	673 ± 33,3	576 ± 21,4	<0,05
24-month-old / 24 месяца	735 ± 34,3	622 ± 38,5	<0,05

Note: represents the average area of 200 neurons. Celiac ganglion data are presented as average values for the right and left nodes.
[a] $p < 0.05$ compared to 60-day-old.
SST-IR – somatostatin immunoreactive; n.s. – not significant.
Примечание: представлена средняя площадь 200 нейронов. Данные по чревному ганглию представлены усредненными значениями по правому и левому узлам.
[a] $p < 0,05$ по сравнению с 60-суточным.
SST-IR – somatostatin immunoreactive, соматостатин иммунореактивные; n.s. – not significant (не значимо).

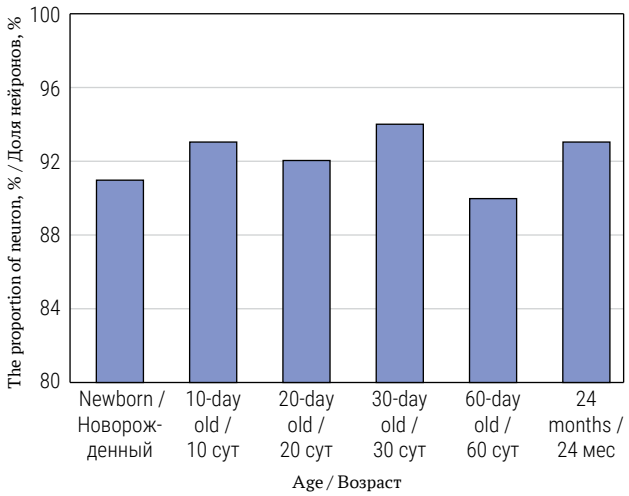

FIG. 2. Percentage of somatostatinergic (SST) neurons of the celiac ganglion colocalizing neuropeptide Y (NPY) in postnatal ontogenesis.
РИС. 2. Процент соматостатинергических (SST) нейронов в чревном ганглии в постнатальном онтогенезе, колокализующих нейропептид Y (NPY).

Our previous data indicate that rats have more SST-IR neurons in nodes of the celiac plexus rather than in other nodes, including the cervicothoracic node [13]. We were the first to discover that the content of SST-IR neurons increases in prevertebral sympathetic nodes (in particular CCG and CMG) in rats during the first 30 days of life. In contrast, their paravertebral cervicothoracic ganglia reach the largest SST-neuronal content immediately after birth (7%) [9].

During postnatal development, the content of SST-IR neurons in this node already decreases to 4% up to Day 10 [9]. Thus, in various sympathetic nodes, the content of SST-IR neurons varies in a heterochronous manner, which may be explained by the features of the target organs innervated. CCG provides sympathetic innervation of the head, the cervicothoracic ganglion of the thorax and neck, and the prevertebral nodes of the organs in the abdominal cavity [16, 17]. We have previously shown that heart-innervating SST-IR neurons in the cervicothoracic node are detected in newborn rat pups only [18].

In this work, we found that there was no change in the content of SST-IR neurons in the prevertebral sympathetic nodes of rats from the early age of 30 days until the age of 2 years. According to the data from the literature sources, the content of SST-IR neurons in the hypothalamus of aged animals (2–2.5 years) also remained constant [19].

We found the average cross-sectional area of SST-IR neurons to be higher in the CG than in the CMG in 20-day-old and older rats. This phenomenon is consistent with the previously discovered size of NPY-IR neurons, which is larger in the CG than in the CMG [8]. We revealed that almost all SST-IR neurons of prevertebral nodes in rats of different ages also express TH and NPY. Neurotransmitters (SST and NPY) suppress peristaltic movements and secretion in the gastrointestinal tract [7]. It is assumed that NPY-IR and SST-negative neurons of the celiac plexus are vasomotor neurons, and NPY/SST-IR neurons are visceromotor neurons [20]. The largest number of visceromotor fibres is directed to intramural nodes in the intermuscular plexus of the gastrointestinal tube [21].

The expression of SST can be altered in pathology. For example, its expression increases in ganglionic neurons of pigs with gastrointestinal inflammation, such as ulcerative colitis and chemically induced descending colon inflammation [22]. There is evidence that SST suppresses inflammatory reactions in the intestine by bidirectional communication between neurons and mast cells and modulates the activity of gut-associated lymphoid tissue [23]. SST stimulates the proliferation of B-lymphoblasts while inhibiting the activity of T cells, granulocyte proliferation, and synthesis of proinflammatory cytokines [24].

SST is also involved in the somatostatinergic anti-inflammatory loop [25], which implies the release of substance P and/or CGRP (calcitonin-gene-related peptide) in the case of damage to peptidergic neurons. Substance P and/or CGRP may induce local neurogenic inflammation, provoking the release of SST from these neurons. Therefore, SST blocks the vicious cycle, preventing excessive release of neuropeptides and thereby initiating inflammation [25].

CONCLUSION

In the early postnatal development of rats, an increase in the content of SST-containing neurons is observed in the paravertebral nodes. This phenomenon provides the formation of sympathetic innervation for the gastrointestinal tract. SST not only inhibits gastrointestinal motility and secretion but also exhibits both analgesic and anti-inflammatory effects. Further experiments examining SST receptors in target organs at different age periods would clarify the features of the sympathetic regulation in the digestive system and could discover more options for the pharmacological management of gastrointestinal diseases.

AUTHOR CONTRIBUTIONS

Andrey I. Emanuilov and Valentina V. Porseva participated in the collection of material, experiments and statistical data processing. Alexei V. Pavlov and Petr M. Masliukov developed the idea and design of the study, and contributed to the writing of the manuscript. All authors approved the final version of the publication.

ВКЛАД АВТОРОВ

А.И. Емануйлов и В.В. Порсева участвовали в сборе материала, проведении экспериментов и статистической обработке данных. А.В. Павлов и П.М. Маслюков разработали идею и дизайн исследования, а также участвовали в написании рукописи. Все авторы утвердили окончательную версию публикации.

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