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EXPERIMENTAL PAPERS

Neurochemical Features of Neuropeptide Y-ergic Enteric Submucosal Neurons in the Rat Small Intestine during Postnatal Ontogenesis

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Abstract—Neuropeptide Y (NPY) performs varied functions in the nervous system, including the regulation of vascular tone and gastrointestinal secretion. Specifically, it exerts a direct inhibitory effect on intestinal motility and secretion. Colocalization of NPY with choline acetyltransferase (ChAT), the enzyme catalyzing acetylcholine synthesis, as well as neuronal NO synthase (nNOS), vasoactive intestinal peptide (VIP) and calcium-binding protein calbindin (CB), was studied in enteric neurons of the submucosal plexus of the rat small intestine at different ages (from the moment of birth until old age) using double immunolabeling and fluorescence microscopy. From the moment of birth, all NPY-ergic neurons colocalize ChAT, while most of them also contain VIP and CB. In aged rats, the percentage of NPY-ergic neurons colocalizing CB, VIP and ChAT decreases. Both in juvenile (since birth until 20 days of age) and aged rats, NPY-ergic neurons were found to express nNOS. Thus, at early stages of ontogeny and in senescence, the rat enteric metasympathetic NPY-ergic submucosal neurons contain a wider range of neurotransmitters compared to adult animals.

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INTRODUCTION

The neurochemical composition of metasympathetic ganglia is highly variable. Acetylcholine, synthesized by the enzyme choline acetyltransferase (ChAT), is found in most ganglionic neurons. In addition to acetylcholine, neurons of intramural ganglia may contain other neurotransmitters, including nitric oxide (NO), serotonin, histamine, as well as neuropeptides, such as neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), and others [1–3].

NPY is widespread in the gastrointestinal tract and occurs in nerve fibers heading to the mucosa and muscularis propria, as well as to vascular smooth muscles [4–6]. NPY occurs in about half of the neurons of the submucosal nerve plexus and only in a small part of neurons in the mouse and

Table 1. Primary antibodies

Type of antibody	Host animal	Dilution	Supplier	Cat No.
choline acetyltransferase (ChAT)*	Goat	1:100	Millipore	AB144p
vasoactive intestinal peptide (VIP)	Rabbit	1:300	Abcam	ab43841
neuronal NO synthase (nNOS)	Rabbit	1:300	Abcam	ab1376
Neuropeptide Y (NPY)	Rabbit	1:500	Abcam	ab30914
NPY	Sheep	1:500	Abcam	ab6173
calbindin (CB)	Rabbit	1:500	Abcam	ab11426

* Antibodies to peripheral ChAT isoform (pChAT).

rat intermuscular plexus [7, 8].

In the intramural enteric ganglia of rodents, most NPY-immunopositive (+) neurons colocalize ChAT [7, 9]. Neurons colocalizing ChAT, calcium-binding protein calbindin (CB), NPY and VIP are considered secretomotor neurons [1, 3, 9]. According to the literature, peripheral neurons contain the so-called peripheral ChAT isoform (pChAT) [10].

NPY has a direct inhibitory effect on intestinal motility and secretion. In addition, NPY promotes neurogenesis and angiogenesis [11–13]. Submucosal neurons regulate the transport of ions and water across the intestinal epithelium, as well as the secretory function of the glands. Secretory disorders in the form of hyper- or hyposecretion may be associated with impaired activity of submucosal NPY-ergic neurons, mainly of the small intestine.

During ontogeny, the morphological characteristics and chemical composition of neurons of the autonomic nervous system undergo changes [2, 14, 15]. This concerns the changes in the size of neurons, levels of calcium-binding proteins, neuropeptides, neurotransmitters and enzymes of their synthesis, including the expression of NPY and its receptors. It was established that the proportion of NPY-ergic neurons in submucosal metasympathetic ganglia of the small intestine increases from the moment of birth to 20–30 days of life, and then declines [8]. Nevertheless, changes in the neurochemical composition of NPY-ergic enteric metasympathetic neurons remain understudied.

This work aimed to determine the colocalization of NPY with enzymes for the synthesis of neurotransmitters as well as other neurotransmitters, in submucosal neurons of the rat small intestine during postnatal ontogeny from the moment of birth to the onset of old age using immunohistochemical methods.

MATERIALS AND METHODS

The experiments were carried out on Wistar rats: newborn and aged 10, 20 and 30 days, 6 months and 2 years (5 animals per age group). Animals were kept under standard vivarium conditions in acrylic cages lined with wood chips, in an acclimatized room (12 h/12 h light/dark cycle, 22 ± 3°C), on a complete balanced diet with ad libitum access to food and water. All the experimental procedures met the "Rules for carrying out animal research work" (order No. 775 of 08/12/1977, Ministry of Health of the USSR), as well as the principles of the Basel Declaration and the recommendations of the Ethics Committee of the Yaroslavl State Medical University (YSMU) (minutes No. 41 of 10/22/2020).

Animals were euthanized with a lethal dose of urethane (3 g/kg, i.p.) and immediately perfused transcardially with a standard 0.01 M PBS (pH 7.4) (Biolot, Russia) followed by (Sigma, USA). After perfusion, duodenal segments (0.5 cm long) were excised and placed into 4% paraformaldehyde/PBS for 1–2 h. A series of 12-μm sections were prepared on a cryostat.

In order to identify neurons containing NPY, ChAT, VIP, nNOS and CB, we used a double immunolabeling technique. Sections were preincubated for 30 min at room temperature in PBS added with 10% donkey serum (Jackson Immunoresearch, USA), 1% Triton X-100, 0.1% bovine serum albumin, and 0.05% thimerosol.

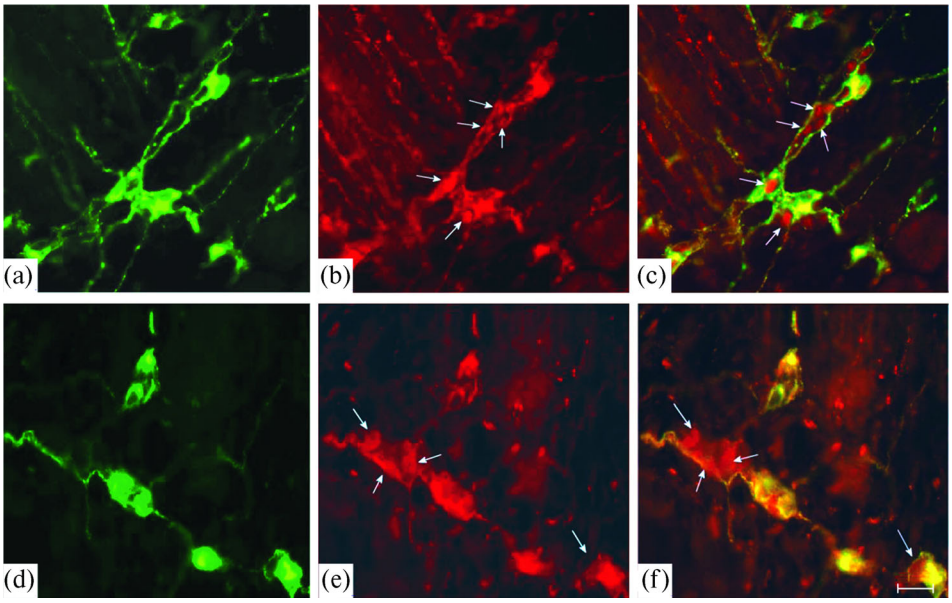

Fig. 1. Micrographs of neurons containing neuropeptide Y (NPY) (a, d), choline acetyltransferase (ChAT) (b, e), and NPY colocalized with ChAT (c, f) in the intramural ganglia of the duodenal submucous plexus of 20-day-old (a–c) and 2-year-old (d–f) rats. ChAT+/NPY– neurons are indicated by arrows. Fluorescence: Cy3 (red, ChAT), FITC (green, NPY). Scale 50 μm.

Next, the sections were incubated with primary antibodies (Table 1) for 24 h at room temperature. After a short wash with PBS, the sections were incubated with secondary antibodies for 2 h. The latter were conjugated to different fluorochromes: fluorescein isothiocyanate (FITC, green fluorescence) and indocarbocyanin (Cy3, red fluorescence) (1 : 150; Jackson Immunoresearch, USA). Thereafter, the sections were rewashed in PBS and embedded in a Vectashield mounting medium for immunofluorescence (Vector Laboratories, USA). The negative control was performed through a replacement of primary antibodies by a donkey serum.

The histological preparations were analyzed on an Olympus BX43 fluorescence microscope (Tokyo, Japan) with an appropriate set of light filters and a cooled Tucsen TCC 6.1ICE CCD digital camera and ISCapture 3.6 software (China). The percentage of immunopositive neurons in digital images of histological preparations was determined using Image J (NIH, USA, http://rsb.info.nih.gov/ij/). The analysis only concerned the nerve cells whose images contained a nucleus with a nucleolus. The total number of immunoreactive neurons containing only red, only green, and both colocalized labels (yellow in overlapped images) was taken as 100%. Immunoreactive neurons were counted across randomly selected measured areas (image area 0.12 mm^2) at 200-fold magnification. For each animal, 5 images were analyzed in 5 sections (one image per section).

The data obtained were statistically processed using Sigma Plot software packages (StatSoft, USA). All values are presented as the arithmetic mean ± standard error of the mean ($M \pm SEM$). The significance of differences in mean values was determined using one-way ANOVA with the Bonferroni correction. Differences were considered statistically significant at $p < 0.05$.

RESULTS

In the submucosal nerve plexus, NPY+ neurons were detected in large numbers in all age groups (Figs. 1–5). All NPY+ neurons (100%), from birth to old age, contained the enzyme for acetylcholine synthesis, ChAT (Fig. 1). At the same time, a part of NPY-immunonegative (–) neurons were also ChAT+, and the percentage of these cells increased significantly from days 20 to 30, as well as in aged rats ($p < 0.05$, Fig. 2).

Since birth, most NPY-containing neurons also

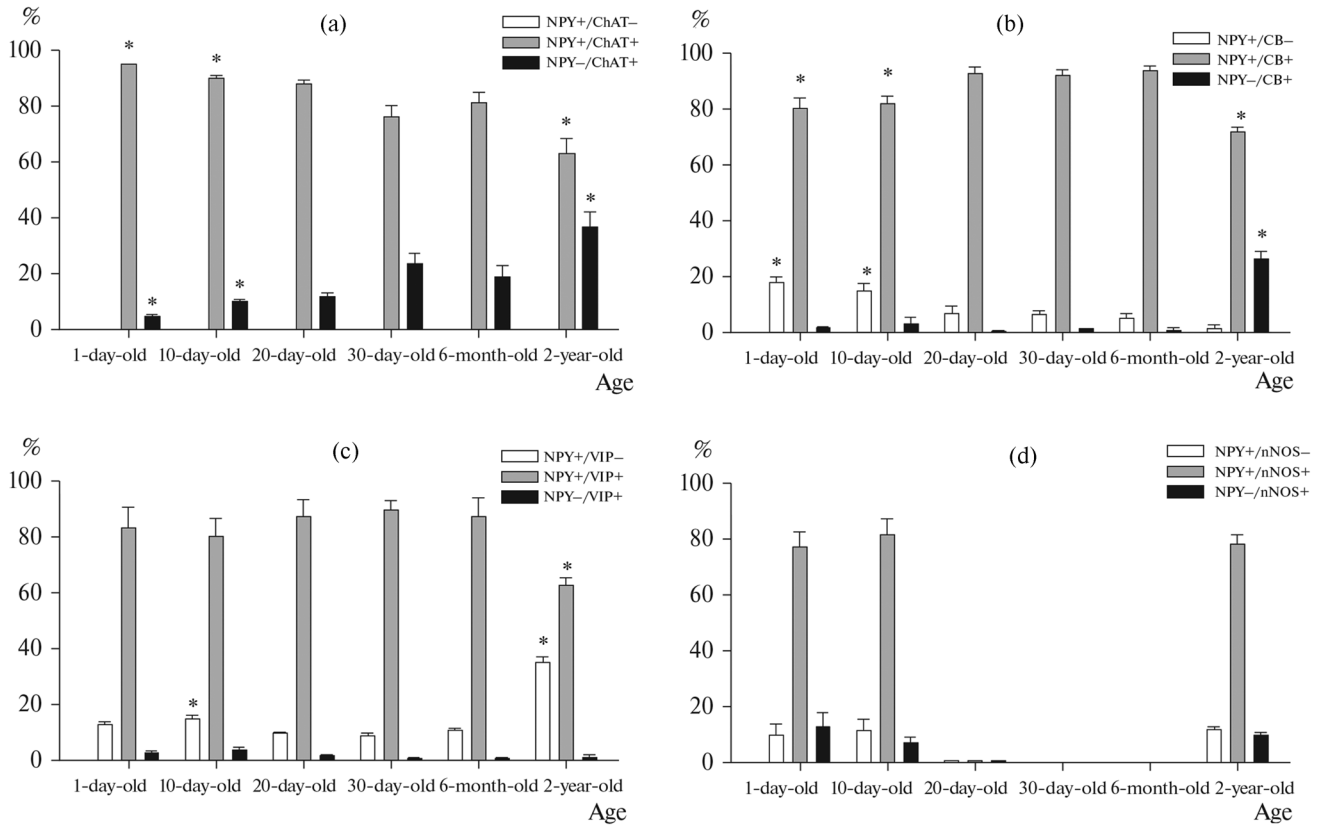

Fig. 2. Percentage of NPY+ neurons colocalizing ChAT (a), calbindin (CB, b), vasoactive intestinal peptide (VIP, c), neuronal NO synthase (nNOS, d) in rats of different ages. *$p < 0.05$. Statistically significant differences vs. 30-day-old rats.

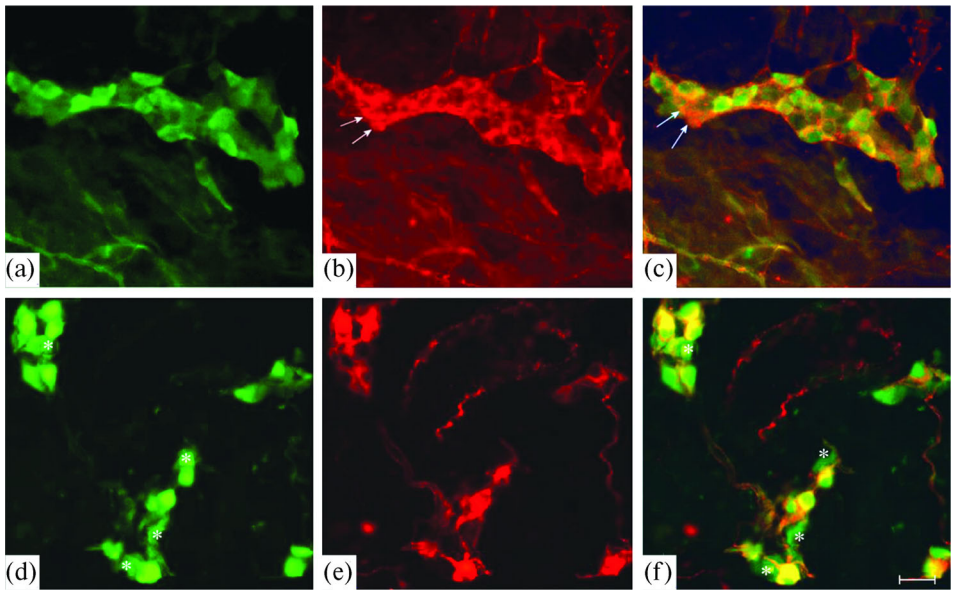

Fig. 3. Micrographs of neurons containing calbindin (CB) (a, d), NPY (b, e) and NPY colocalized with CB (c, f) in duodenal submucosal intramural ganglia of 20-day-old (a–c) and 2-year-old (d–f) rats. CB–/NPY+ neurons are indicated by arrows, CB+/NPY– neurons are indicated by asterisks. Fluorescence: Cy3 (red, NPY), FITC (green, CB). Scale 50 microns.

contained CB (Fig. 3), VIP (Fig. 4), and nNOS (Fig. 5). The percentage of NPY+ neurons colo-

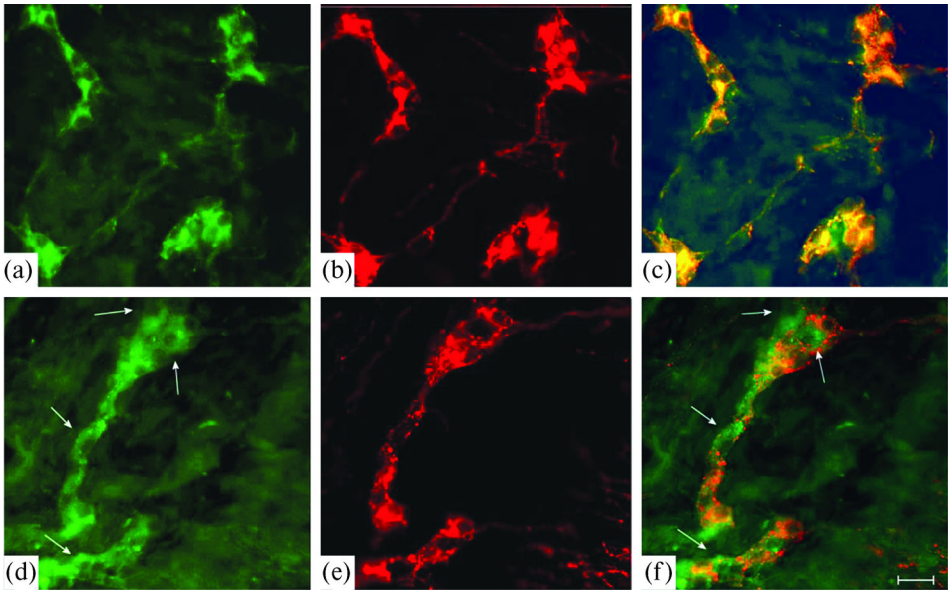

Fig. 4. Micrographs of neurons containing NPY (a, d), VIP (b, e), and NPY colocalized with VIP (c, f) in duodenal submucosal intramural ganglia of 20-day-old (a–c) and 2-year-old (d–f) rats. NPY+/VIP– neurons are indicated by arrows. Fluorescence: Cy3 (red, VIP), FITC (green, NPY). Scale 50 microns.

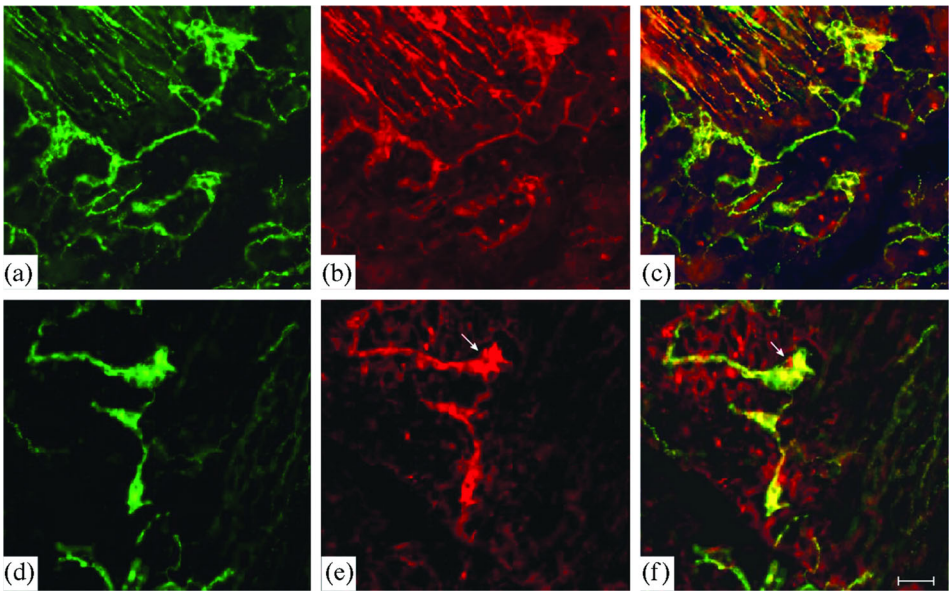

Fig. 5. Micrographs of neurons containing NPY (a, d), nNOS (b, e) and NPY colocalized with nNOS (c, f) in duodenal submucosal intramural ganglia of 10-day-old (a–c) and 2-year-old (d–f) rats. nNOS+/NPY– neuron is indicated by arrow. Fluorescence: Cy3 (red, nNOS), FITC (green, NPY). Scale 50 microns.

calizing CB significantly increased between days 10 and 20 of postnatal life ($p < 0.05$, Fig. 2), remaining constant up to 6 months, and decreased in aged rats ($p < 0.01$). In aged vs. younger rats, the percentage of CB+ neurons containing no NPY also increased significantly ($p < 0.001$).

The percentage of NPY+ neurons colocalizing VIP was not statistically indistinguishable in juvenile vs. adult rats ($p > 0.05$, Fig. 2), but significantly decreased in aged rats ($p < 0.001$). In aged vs. younger animals, the proportion of NPY+/VIP– neurons significantly increased ($p < 0.001$).

In young rats, since birth to 20 days of life, as

well as in aged animals, nNOS was detected in NPY+ neurons. It is noteworthy that while a significant number of nNOS+ neurons were detected in newborn and 10-day-old animals, only sparse cells were observed in 20-day-old rats. Nevertheless, in aged rats, the proportion of nNOS+ neurons again becomes comparable to that in newborns and 10-day-olds, with no significant differences between these groups ($p > 0.05$, Fig. 2).

DISCUSSION

Our results show that NPY+ neurons are detected in submucosal nodes in large numbers since the very moment of birth. The data of our previous study indicate that the percentage of NPY+ submucosal neurons of the small intestine varies during ontogeny, peaking in rats at the age of 20–30 days [8].

NPY+ and NPY– neurons belong to different functional populations. The submucosal nerve plexus in guinea pigs and mice comprises four types of neurons, including secretomotor and vasomotor neurons, as well as their own primary afferent neurons [1, 3]. Neurons that colocalize ChAT, CB, NPY, and VIP are considered secretomotor neurons [9].

NPY exerts a direct inhibitory effect on intestinal motility and secretion. The inhibitory effect of NPY on the intestinal secretory function is implemented through activation of postsynaptic Y1 receptors of enterocytes and neuronal presynaptic Y2 receptors [4, 16]. Given that submucosal neurons are involved in the regulation of secretion, it can be assumed that at the age of 20–30 days, the secretory function of the small intestine undergoes an ultimate formation associated with the transition from milk to independent nutrition. At the same time, NPY can play a special role in the development of the small intestine's function, acting not only as a co-transmitter, but also as a trophic factor. Apart from affecting vascular tone, cardiac performance, secretory and motor functions of the gastrointestinal tract, NPY stimulates neurogenesis and also has trophic effects, specifically, promoting angiogenesis and myocardial hypertrophy [11–13]. In the gut, NPY also plays an important role, modulating the functions of immune cells and the epithelial barrier.

Here we addressed for the first time NPY and ChAT colocalization in rats from birth to old age. During postnatal ontogeny, all NPY+ neurons contain the enzyme for acetylcholine synthesis, ChAT, which is consistent with the literature data obtained on adult animals [1, 3, 7]. We also found that NPY+ cholinergic neurons predominantly contain VIP and CB in juvenile, adult, and aged rats.

Interestingly, one and the same neuron of the submucosal nerve plexus contains neurotransmitters that both stimulate (VIP) and inhibit (NPY) intestinal secretion [1]. Apparently, the release of VIP or NPY relies on the type of stimulation. For example, noradrenaline is released from sympathetic terminals at a low-frequency stimulation, while NPY is released by high-frequency stimulation [5]. NPY is believed to have a pro-inflammatory effect, while VIP is anti-inflammatory [17]. An increase in the percentage of NPY+/VIP– and a decrease in the percentage of VIP+ neurons may indicate that aging is accompanied by an increase in the level of inflammatory processes in many tissues, including the nervous system and gastrointestinal tract [18].

We found that, at an early age, most NPY+ neurons transiently express nNOS. Moreover, a small part of NPY– neurons in newborn and 10-day-old animals also colocalize nNOS. Nevertheless, nNOS in the submucosal plexus is detected only in single neurons at the age older than 20 days, however, again becomes detectable in aged animals. According to the literature data, only 1% of submucosal neurons of the small intestine in an adult mouse contain nNOS, whereas in the late embryonic and early postnatal period, nNOS occurs in 50% of submucosal neurons [19, 20]. Similarly, cholinergic neurons of sympathetic ganglia express ChAT and the enzyme for the synthesis of catecholamines, tyrosine hydroxylase, which ceases to be detected since the third week of life [21]. Also, transient nNOS expression is observed during the embryonic period in the spinal nodes, cerebellum, brainstem, cerebral cortex, and hippocampus [22, 23]. Some authors relate this transient expression with the role of NO in eliminating excess synaptic innervation, which is observed in the developing nervous system, as well

as with fine tuning of the synaptic apparatus, accompanied by the activation of some synapses and the elimination of low-activity ones [23, 24]. Also, NO increases the excitability of neurons by modulating the activity of K^+ channels [24]. An increase in the expression of nNOS in old age is also observed in CNS neurons [25, 26]. It is assumed that this may, on the one hand, promote apoptosis, and on the other hand, have an anti-apoptotic significance.

Thus, at early stages of ontogeny and in old age, enteric neurons of the metasympathetic nervous system, specifically those expressing neuropeptide Y, contain a wider spectrum of neurotransmitters as compared to adult animals. In this study, it was shown for the first time that NPY-ergic neurons of the rat submucosal plexus, along with choline acetyltransferase, vasointestinal peptide and calbindin, in newborn and aged rats, also express neuronal NO synthase.

AUTHORS' CONTRIBUTION

Conceptualization and experimental design: P.M.M. and A.V.P.; data collection: A.F.B. and P.A.V.; data processing: A.F.B. and P.A.V.; writing and editing a manuscript: P.M.M. and A.V.P.

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CONFLICT OF INTEREST

The authors declare that they have neither evident nor potential conflict of interest related to the publication of this article.

REFERENCES

1. Furness JB (2006) The enteric nervous system. Blackwell Publishing, Oxford.
2. Masliukov PM, Budnik AF, Nozdrachev AD (2017) Neurochemical Features of Metasympathetic System Ganglia in the Course of Ontogenesis. Adv Gerontol 7(4):281-289. https://doi.org/10.1134/S2079057017040087
3. Furness JB, Stebbing MJ (2018) The first brain: Species comparisons and evolutionary implications for the enteric and central nervous systems. Neurogastroenterol Motil 30(2):e13234. https://doi.org/10.1111/nmo.13234
4. Cox HM (2007) Neuropeptide Y receptors; antisecretory control of intestinal epithelial function. Auton Neurosci 133(1):76–85. https://doi.org/10.1016/j.autneu.2006.10.005
5. Nozdrachev AD, Masliukov PM (2011) Neuropeptide Y and autonomic nervous system. Zh Evol Biokhim Fiziol 47:105-112. https://doi.org/10.1134/S0022093011020010
6. Rytel L, Szymanska K, Gonkowski I, Wojtkiewicz J (2018) Neurochemical characterization of intramural nerve fibres in the porcine oesophagus. Anat Histol Embryol 47(6):517-526. https://doi.org/10.1111/ahe.12391
7. Mongardi Fantaguzzi C, Thacker M, Chiocchetti R, Furness JB (2009) Identification of neuron types in the submucosal ganglia of the mouse ileum. Cell Tissue Res 336(2):179-189. https://doi.org/10.1007/s00441-009-0773-2
8. Budnik AF, Aryaeva D, Vyshnyakova P, Masliukov PM (2020) Age related changes of neuropeptide Y-ergic system in the rat duodenum. Neuropeptides 80:101982. https://doi.org/10.1016/j.npep.2019.101982
9. Mann PT, Furness JB, Southwell BR (1999) Choline acetyltransferase immunoreactivity of putative intrinsic primary afferent neurons in the rat ileum. Cell Tissue Res 297:241–248. https://doi.org/10.1007/s004410051352
10. Kolos EA, Korzhevskii DA (2016) Heterogeneous choline acetyltransferase staining in cholinergic neurons. Neurochem J 10(1):47-52 (In Russ).
11. Jia C, Hegg CC (2015) Effect of IP3R3 and NPY on age-related declines in olfactory stem cell proliferation. Neurobiol Aging 36(2):1045-1056. https://doi.org/10.1016/j.neurobiolaging.2014.11.007
12. Saraf R, Mahmood F, Amir R, Matyal R (2016) Neuropeptide Y is an angiogenic factor in cardiovascular regeneration. Eur J Pharmacol 776:64-70. https://doi.org/10.1016/j.ejphar.2016.02.033
13. Tan CMJ, Green P, Tapoulal N, Lewandowski AJ, Leeson P, Herring N (2018) The Role of Neuropeptide Y in Cardiovascular Health and Disease. Front Physiol 9:1281. https://doi.org/10.3389/fphys.2018.01281
14. Foong JP (2016) Postnatal Development of the Mouse Enteric Nervous System. Adv Exp Med Biol 891:135-143. https://doi.org/10.1007/978-3-319-27592-5_13
15. Masliukov PM, Moiseev K, Budnik AF, Nozdrachev AD, Timmermans JP (2017) Devel-

opment of calbindin- and calretinin-immunopositive neurons in the enteric ganglia of rats. Cell Mol Neurobiol 37(7):1257–1267. https://doi.org/10.1007/s10571-016-0457-x
16. Tough IR, Forbes S, Tolhurst R, Ellis M, Herzog H, Bornstein JC, Cox HM (2011) Endogenous peptide YY and neuropeptide Y inhibit colonic ion transport, contractility and transit differentially via Y1 and Y2 receptors. Br J Pharmacol 164:471–484. https://doi.org/10.1111/j.1476-5381.2011.01401.x
17. Chandrasekharan B, Nezami BG, Srinivasan S (2013) Emerging neuropeptide targets in inflammation: NPY and VIP. Am J Physiol Gastrointest Liver Physiol 304(11):G949-G957. https://doi.org/10.1152/ajpgi.00493.2012
18. Wyss-Coray T (2016) Ageing, neurodegeneration and brain rejuvenation. Nature 539(7628):180-186. https://doi.org/10.1038/nature20411
19. Young HM, Ciampoli D (1998) Transient expression of neuronal nitric oxide synthase by neurons of the submucous plexus of the mouse small intestine. Cell Tissue Res 291:395–401. https://doi.org/10.1007/s004410051009
20. Bergner AJ, Stamp LA, Gonsalvez DG, Allison MB, Olson DP, Myers MG Jr, Anderson CR, Young HM (2014) Birthdating of myenteric neuron subtypes in the small intestine of the mouse. J Comp Neurol 522(3):514-527. https://doi.org/10.1002/cne.23423
21. Maslyukov PM, Shilkin VV, Timmermans JP (2006) Immunocytochemical characteristics of neurons in the stellate ganglion of the sympathetic trunk in mice during postnatal ontogenesis. Neurosci Behav Physiol 36(8):851–855. https://doi.org/10.1007/s11055-006-0097-6
22. Bredt DS, Snyder SH (1994) Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. Neuron 13:301-313. https://doi.org/10.1016/0896-6273(94)90348-4
23. Portillo F, Moreno-López B (2020) Nitric oxide controls excitatory/inhibitory balance in the hypoglossal nucleus during early postnatal development. Brain Struct Funct 225(9):2871-2884. https://doi.org/10.1007/s00429-020-02165-9
24. González-Forero D, Moreno-López B (2014) Retrograde response in axotomized motoneurons: nitric oxide as a key player in triggering reversion toward a dedifferentiated phenotype. Neuroscience 283:138-165. https://doi.org/10.1016/j.neuroscience.2014.08.021
25. Dawson TM, Dawson VL (2018) Nitric oxide signaling in neurodegeneration and cell death. Adv Pharmacol 82:57-83. https://doi.org/10.1016/bs.apha.2017.09.003
26. Moiseev KY, Vishnyakova PA, Porseva VV, Masliukov AP, Spirichev AA, Emanuilov AI, Masliukov PM (2020) Changes of nNOS expression in the tuberal hypothalamic nuclei during ageing. Nitric Oxide 100-101:1-6. https://doi.org/10.1016/j.niox.2020.04.002

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