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Neurotransmitter Composition of Neurons in the Cranial Cervical and Celiac Sympathetic Ganglia in Postnatal Ontogenesis

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The neurotransmitter composition of neurons in the cranial cervical ganglion (CCG) and celiac ganglia (CG) in rats of different ages (neonatal, 10, 12, 30, and 60 days) was studied by immunohistochemical methods. The results showed that most neurons in these sympathetic ganglia contain tyrosine hydroxylase (TH). Most TH-positive neurons were also neuropeptide Y (NPY)-positive. In all ganglia, the proportions of neurons containing NPY increased from the moment of birth to the end of the first month of life. In the CG, NPY was present in a significantly greater proportion of neurons than in the CCG. Substance P, vasoactive intestinal peptide, and choline acetyltransferase were present in occasional neurons in the CCG and CG from birth. There was no change in the proportion of this type of neuron with age. Definitive establishment of the neurotransmitter composition in the sympathetic ganglia studied here occurred by the end of the first month of life.

KEY WORDS: sympathetic nervous system, cranial cervical ganglion, celiac ganglia, immunohistochemistry, ontogenesis.

Published data provide evidence that many neurons simultaneously contain several neurotransmitters – up to six in a single cell [9]. Catecholaminergic neurons have been found to contain somatostatin, neuropeptide Y (NPY), opioid peptides, and galanin. Neurons not containing catecholamines contain vasoactive intestinal peptide (VIP), calcitonin gene-related peptide, NPY, and substance P. Most noradrenergic neurons contain NPY [6, 9, 12].

During postnatal ontogenesis, sympathetic ganglion neurons show a rearrangement in their neurotransmitter composition, which may occur under the influences of a variety of trophic factors [8, 10, 11]. Catecholamines have been shown to be present in primary sympathetic ganglia during the embryonic period [7, 8]. In the ganglia of the solar plexus of guinea pigs and the paravertebral ganglia of rats, tyrosine hydroxylase (TH), along with NPY, is detected at the early embryonic stages [4, 14], before the moment at which contact is made with target organs or preganglionic neurons.

Previous studies of the stellate ganglion in rodents showed that from birth, the greater proportion of neurons contain the catecholamine synthesis enzyme TH. Most choline acetyltransferase (CAT)-positive neurons in neonates and 10-day-old rats were also TH-positive, while only occasional cells in 30- and 60-day rats contained both enzymes. The proportions of cells containing TH and NPY increased from birth and at all the ages studied. In addition, there was a reduction in the relative content of somatostatin-positive neurons. The proportion of VIP-positive cells and neurons containing CAT increased to day 10 of life and then decreased. Maturation of the set of neurotransmitters in the stellate ganglion in rats and mice was also shown to be complete by the end of the second month of life [2, 3, 11].

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TABLE 1. Primary Antibodies

Antibody to	Source animal	Dilution	Source
Substance P	Rabbit	1:1000	Chemicon (USA)
TH	Rabbit	1:500	Biotrend (Germany)
VIP	Rabbit	1:400	Affinity (UK)
NPY	Rabbit	1:500	Chemicon (USA)
CAT	Goat	1:200	Chemicon (USA)

Note. TH is tyrosine hydroxylase; VIP is vasoactive intestinal peptide; NPY is neuropeptide Y; CAT is choline acetyltransferase.

However, no comparative analyses of the development of neurons in other sympathetic ganglia during ontogenesis have been reported.

The aim of the present work was to identify the immunohistochemical characteristics of neurons in the cranial cervical (CCG) and celiac (CG) ganglia in rats during postnatal ontogenesis.

MATERIALS AND METHODS

Studies were performed using neonatal rat pups and rat pups aged 10 and 12 days and one and two months (five individuals of each age). After administration of lethal doses of sodium pentobarbital (Nembutal®, 300 mg/kg, i.p.), animals were subjected to transcardiac perfusion with isotonic saline containing heparin followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4). After perfusion, the cranial cervical and celiac ganglia on each side were harvested and placed in the same fixative solution used for perfusion for 1–2 h. Serial sections of thickness 12 μm were cut using a cryostat. Neurons containing TH, CAT, VIP, NPY, and substance P were detected using labeled antibodies (Table 1).

Sections were preincubated for 30 min at room temperature in phosphate-buffered saline (PBS) supplemented with 10% serum, 1% Triton X-100, and 0.1% bovine serum albumin. Sections were then incubated with primary antibodies for 24 h at room temperature. After brief washing with PBS, sections were incubated with secondary antibodies conjugated with fluorochrome, i.e., fluorescein isothiocyanate (Jackson Immuno Research Lab., USA) for 2 h (at a dilution of 1:1000), which gives a green fluorescence.

The relative contents of immunopositive neurons, in addition to labeling for individual neurotransmitters, were determined by labeling the entire neuron population with another fluorochrome, i.e., Neuro Trace (Molecular Probes, USA), which gives a red fluorescence. After washing with PBS, sections were incubated in this solution for 20 min (at a dilution of 1:200).

Further analysis of sections was performed using a version 12 LOMO Mikmed 2 fluorescence microscope (LOMO, Russia) fitted with the appropriate set of light filters and a CCD camera. Images were analyzed using computer programs. The sizes and percentage compositions of immunopositive neurons were determined using the three central sections from each ganglion, separated by about 0.05 mm. Counts included only neurons with clearly identifiable nuclei. The proportions of immunoreactive neurons were calculated as the ratios of neurons immunoreactive to the marker of interest on the section to the total number of neurons on the section, multiplied by 100. Neuron cross-sectional areas were determined using the non-commercial program Image J (NIH, USA). This parameter was measured in a random set of neurons immunopositive for each of the markers in each age group as described by Avtandilov [1].

Statistical analysis included calculation of arithmetic means and standard errors of the mean. Significant differences were identified using Student's t test. Differences were regarded as significant at $p < 0.05$.

RESULTS

There were no significant differences in the distributions or morphometric characteristics of neurons immunoreactive for different markers in the CCG or CG on the right and left sides. Within ganglia, neurons of different populations were distributed diffusely. The greatest numbers of neurons in the CCG and CG at all ages were noradrenergic and contained the noradrenaline synthesis enzyme TH. The relative contents of these neurons during early postnatal ontogenesis showed virtually no change, and ranged from 88 ± 5% to 94 ± 4%.

Large proportions of TH-positive neurons in the CCG and CG were also NPY-positive (Fig. 1). The proportions of neurons in the CCG and CG containing TH and NPY increased from birth to the end of the second month of life in all the ganglia studied (Fig. 2). In the CG, a significantly

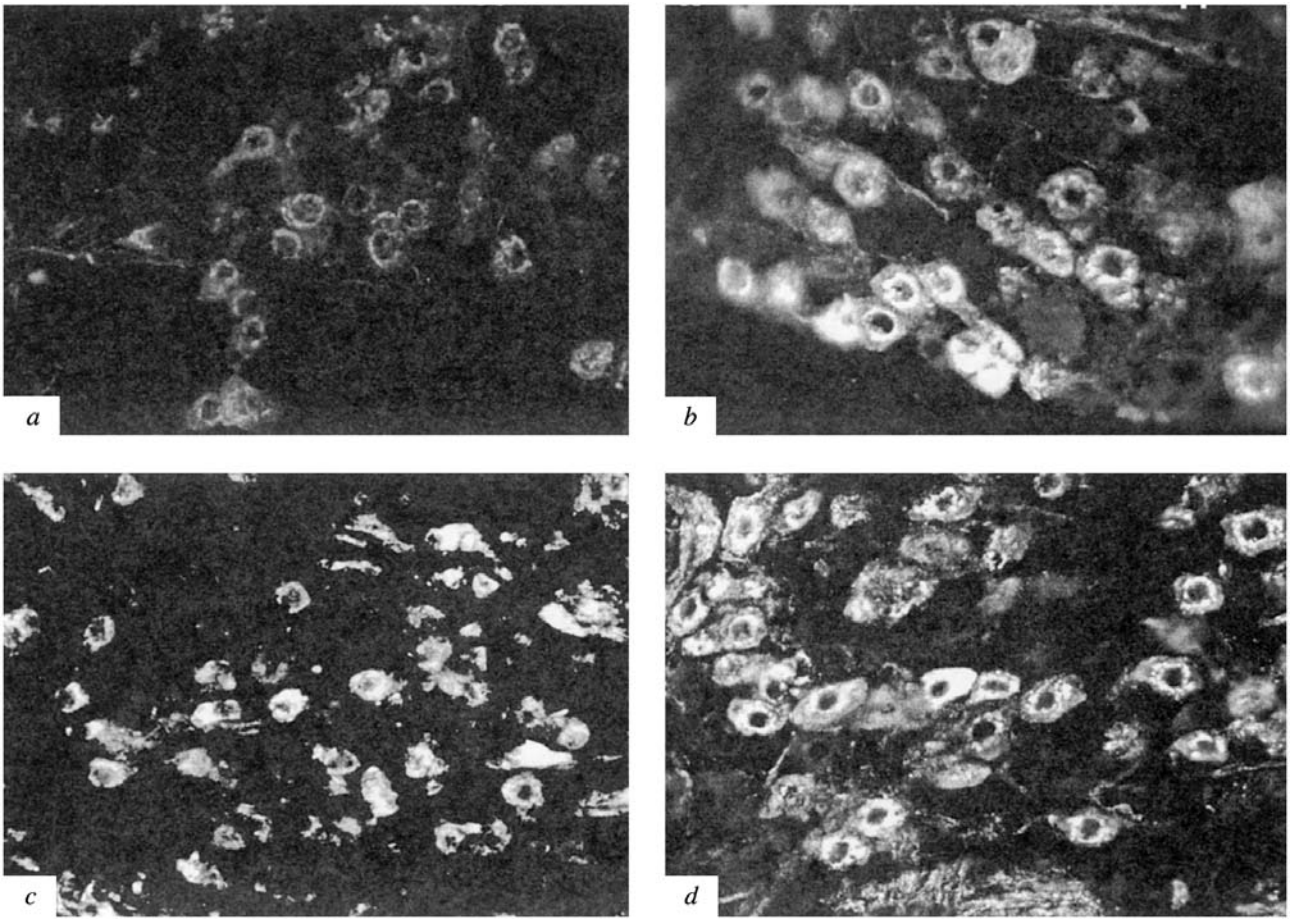

Fig. 1. Neurons containing neuropeptide Y in the celiac (*a*, *b*) and cranial cervical (*c*, *d*) ganglia in neonatal (*a*, *c*) and 30-day-old (*b*, *d*) rat pups. Fluorescein-isothiocyanate fluorescence. Objective ×10; ocular ×10.

greater proportion of neurons contained NPY than in the CCG. Only occasional cells in the CCG and CG were immunopositive for NPY and negative for TH.

Substance P, VIP, and CAT were seen in occasional neurons in the CCG and CG from birth. There were no changes in the proportions of these neurons with age. All VIP-positive cells also contained CAT.

Analysis of morphometric characteristics showed that neurons containing different neurotransmitters had different mean cross-sectional areas. Neurons positive for substance P in the CCG and CG in all rat pups were significantly smaller in size ($p < 0.01$) (Fig. 3). Neurons containing VIP and CAT had significantly larger mean cross-sectional areas in the CCG ($p < 0.05$). In the CG, the mean cross-sectional areas of TH-, VIP-, and CAT-positive neurons did not differ significantly ($p > 0.05$). In all age groups, the mean cross-sectional areas of neurons containing NPY in the CCG and CG were significantly smaller than those of neurons containing TH ($p < 0.05$).

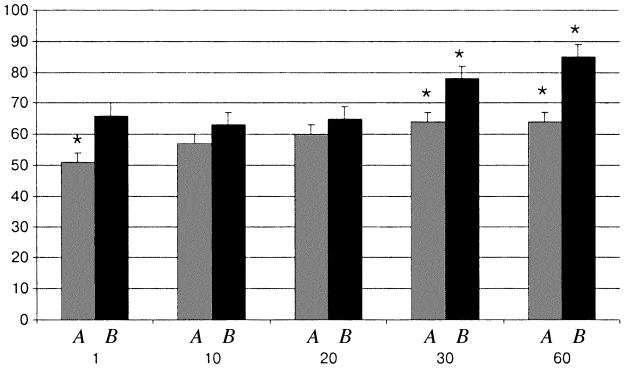

Fig. 2. Relative contents of neurons expressing neuropeptide Y in the cranial cervical and celiac ganglia of rat pups of different ages. The horizontal axis shows rat pup age (days); *A*) cranial cervical ganglion; *B*) celiac ganglion; the ordinate shows the study parameter (%). *Significant differences compared with data from the same ganglia in 10-day-old animals, $p < 0.05$; vertical bars show standard errors.

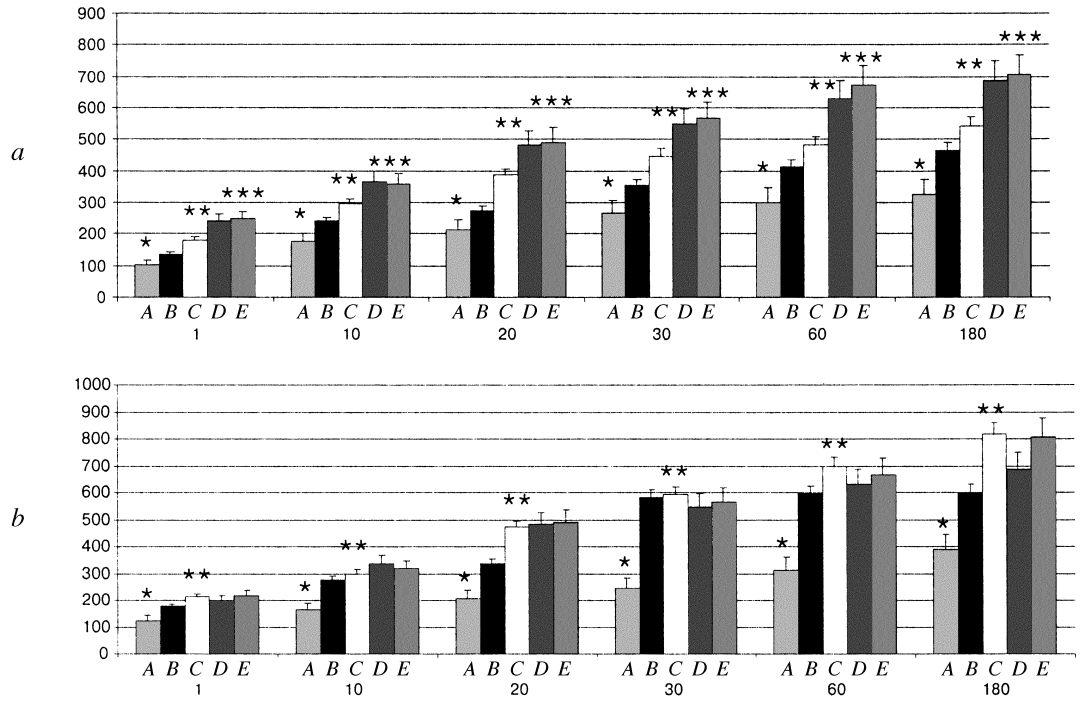

Fig. 3. Cross-sectional areas of neurons immunoreactive for substance P (SP), neuropeptide Y (NPY), tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP), and choline acetyltransferase (CAT) in the cranial cervical ganglion (*a*) and celiac ganglion (*b*) in rat pups of different ages. The horizontal axis shows: *A*) SP-positive neurons; *B*) NPY-positive neurons; *C*) TH-positive neurons; *D*) VIP-positive neurons; *E*) CAT-positive neurons; and age (days); the ordinate shows the study parameter (μm^2); *significant difference compared with the groups of SP and NPY positive neurons; **significant differences between the groups of TH- and NPY-positive neurons; ***significant differences between the groups of TH-positive neurons and cholinergic neurons (VIP- and CAT-positive); $p < 0.05$; vertical bars show standard errors of the mean.

The mean cross-sectional area of NPY- and TH-positive neurons in the CCG was significantly smaller than that in the CG ($p < 0.01$).

DISCUSSION

The results obtained here, along with previously reported data from studies of the stellate ganglion (SG) [2, 3], provide evidence that most neurons in the sympathetic para- and prevertebral ganglia studied are catecholaminergic and contain TH at birth. A large proportion of TH-positive neurons in these ganglia were also NPY-positive. Subsequently, as age increases, the proportion of these neurons increases. Increases in the proportion of NPY-containing catecholaminergic neurons in postnatal ontogenesis are seen in several mammal species, including humans [13].

Some noradrenergic neurons also contain other neurotransmitters. A small proportion of sympathetic cholinergic neurons contain VIP and CAT and innervate the sweat glands and periosteum [5]. Unlike our previous data from studies of the SG [2, 3], the proportions of VIP- and CAT-containing neurons in the CCG and CG were very low and did not change with age. The differences in the dynamics of changes in the percentage composition of cholinergic neurons in the CCG, CG, and SG are probably associated with the fact that neurons in the para- and prevertebral sympathetic ganglia send their fibers to different target organs.

Nonetheless, cholinergic neurons are present in various sympathetic ganglia by the moment of birth. This contradicts the previous view that cholinergic neurons are initially catecholaminergic, switching of neurotransmitter composition occurring during the first two weeks following birth in response to trophic factors released by the target organs [5].

Neurons in sympathetic ganglia containing different neurotransmitters have different cross-sectional areas. Unlike the stellate ganglion [11], NPY-positive neurons in the CCG and CG had smaller mean cross-sectional areas than TH-containing neurons. The populations of substance P-positive neurons in the CCG and CG consist of small cells.

These data supplement our knowledge of the postnatal development of the neuronal organization of the autonomic nervous system, particularly the inhomogeneity of the cell composition of neurons in the stellate ganglia of cats and rodents at the moment of birth [2, 3]. There are individual neuron populations differing in terms of their locations,

sizes, histochemical, and functional characteristics. The set of neurotransmitters typical of the adult organism is present in neurons in rat pup sympathetic ganglia from the moment of birth, and the development of neuron populations containing different neurotransmitters in the CCG, SG, and CG occurs at different times. Ultimately, the neurotransmitter composition of ganglion neurons stabilizes by the end of the first month of life.

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