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EXPERIMENTAL ARTICLES

Heterogeneous Choline Acetyltransferase Staining in Cholinergic Neurons

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Abstract—We studied the features of choline acetyltransferase (ChAT) distribution in cholinergic structures of the central and peripheral nervous systems; specifically in the spinal cord and dorsal root ganglion (DRG) of embryo (E20), newborn, or adult rats using a goat polyclonal antibody. We found that this anti-ChAT antibody selectively labels both central and peripheral cholinergic structures. We observed cholinergic neurons with weak immunoreactivity. We demonstrated that at all stages of ontogeny that we studied, four groups of cholinergic neurons occurred in the cervical part of the spinal cord, viz., the small neurons of the dorsal horns, neurons of Rexed's lamina X, neurons of the area of Rexed's laminae VI–VII, and cells of the ventral horns. Interneurons of Rexed's laminae VI–VII exhibited maximum ChAT reactivity as compared to other groups of cholinergic neurons of the spinal cord at all developmental stages we studied. We found an increase in the number of immunopositive cells of lamina X from E20 stage to the early postnatal and mature stages. In the peripheral cholinergic structures, i.e., the DRG, of the rat, all neurons expressed ChAT at all stages of ontogeny.

Keywords: cholinergic systems, immunohistochemistry, spinal cord, dorsal root ganglion, cholinacetyltransferase

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INTRODUCTION

Cholinergic neurons produce acetylcholine (ACh) as a neurotransmitter, which plays an important role in the regulation of the functions of the central and peripheral nervous systems. ACh is involved in various neuropsychic functions, including learning, memory, and sleep. In the spinal cord, the cholinergic system participates in motor functions [1–5]. In the peripheral nervous system, cholinergic neurons are involved in the control of autonomic functions and play a key role in the regulation of gastrointestinal motility [6, 7]. Until now, there have been no methods for the direct visualization of ACh in neurons. Morphological studies of cholinergic cells are usually performed using the immunocytochemical detection of the vesicular acetylcholine transporter protein [8–10] or the choline acetyltransferase (ChAT) enzyme. The later protein is a key enzyme of neurotransmitter synthesis from acetyl-coenzyme A and intracellular choline [11]. To date, ChAT is considered as the most selective marker of cells that produce ACh [12–14]. However, there are no modern data on the formation of cholinergic neurons in the central (CNS) or peripheral nervous systems (PNS) in ontogeny reported using this marker. The spectrum of anti-ChAT antibodies is rather wide; however, not all of them are suitable for immunocytochemistry. Selection of a reagent determines the specificity of the immunohistochemical reaction, the intensity of staining of neurons and their processes, and the assessment of the enzyme location in the cell and distribution of ChAT in the CNS and PNS. Application of various antibodies often results in the generation of controversial data. The goat polyclonal antibody is most popular one for the study of cholinergic structures in the CNS [13, 15–17].

The aim of the present study was to examine the ChAT distribution in cholinergic structures with clear neurotransmitter specificity in the spinal cord and dorsal root ganglion of embryo, newborn, and adult rats using immunohistochemistry and confocal laser microscopy.

MATERIALS AND METHODS

In the present study, we used spinal cord and dorsal root ganglia samples from 20-day-old ($n = 5$), newborn ($n = 8$), and adult 3–4-month-old ($n = 5$) Wistar rats. The animals were housed and the experimental procedures were performed in accordance with the Rules for work with experimental animals (Order no. 755 from August 12, 1977 of the Ministry of Health Care of the Soviet Union). The rats were euthanized according to the international rules of the Declaration of Helsinki. The cervical part of the spinal cord with

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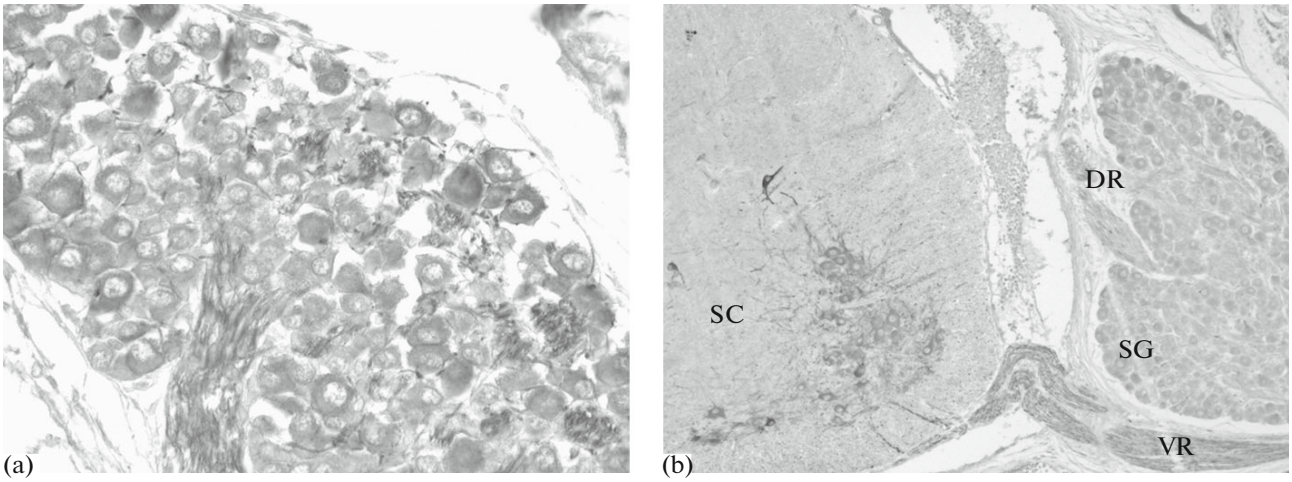

Fig. 1. Immunohistochemical staining of choline acetyltransferase in (a) cells of the developing spinal cord ganglion of an E20 rat embryo and (b) cells of the spinal cord and sensory ganglion of a newborn rat. SC, spinal cord; SG, sensory ganglion; DR, fibers of the dorsal root of the spinal cord; VR, fibers of the ventral root of the spinal cord. Magnification: (a) ×400; (b) ×100.

adjacent sensory dorsal root ganglia of embryo, newborn, and adult rats were sampled on the level of C3–C5 segments and used as an object for the study. The samples were fixed with a zinc–ethanol–formaldehyde fixative for 1 day [18], dehydrated in alcohol with ascending concentration, and embedded in paraffin. Serial 5-μm-thick sections were prepared for immunohistochemical studies according to the generally accepted method. Prior to ChAT immunohistochemistry, we performed heat antigen retrieval. For ChAT detection we used the primary goat polyclonal antibody (Merck Millipore, Chemicon, AB144, United States) diluted at 1 : 250 [13]. Rabbit anti-goat biotinylated secondary antibody (Dako, Denmark) diluted at 1 : 200 and streptavidin–peroxidase (Spring Bioscience, United States) were used for detection of primary antibody. The reacted antibodies were visualized using diaminobenzidine (DAB+, Dako, Denmark). The sections were placed under a cover slip with Cytoseal 60 medium (Germany). Images were captured and studied using a Leica DM 750 microscope and an ICC 50 camera (Leica, Germany). The images were analyzed using LAS EZ computer software (Leica, Germany), which allowed to estimate the cell size. Spinal cord preparations from adult rats were studied using confocal laser microscopy. For this purpose, we visualized antibodies that reacted with ChAT using a streptavidin–Cy5 conjugate (Invitrogen, United States) diluted as 1 : 80; the sections were placed under a cover slip with Fluorescence Mounting Medium (Dako, Denmark). The preparations were examined and images were captured using an LSM710 confocal microscope (Zeiss, Germany). The images were analyzed using ZEN2012 software (Zeiss, Germany). The confocal microscope was equipped with a C-Apochromat 63× lens with water immersion. Fluorescence of Cy5 was excited by a helium-neon laser with the wavelength of 633 nm. Immunopositive structures were delineated using a rat spinal cord atlas [19].

RESULTS

In the spinal cord of E20 rat embryos, ChAT-containing structures were observed in the ventral and dorsal horns and formed the intermediate and central gray matter. Single immunopositive neuroblasts of the deep layers of the spinal cord dorsal roots were fusiform or oval. In the intermediate gray matter, we observed stellar intensely stained cells with long immunopositive branching processes, which were oriented dorsoventrally and mediolaterally. The ChAT containing cells of this region were 12 × 13 μm in size. Immunopositive cells of Rexed's lamina X were weakly stained. The cells were oval and their stained processes were oriented mediolaterally. Developing cholinergic motoneurons of the ventral horns of the spinal cord had immunopositve processes, which formed a network that extended to the white matter of the ventral roots. In some cells, we observed sparse intensely stained round structures on the bodies, which probably were axosomatic synapses. At this stage of ontogeny, cells of the intermediate gray matter exhibited the most intense immunohistochemical ChAT reactivity in the cervical part of the spinal cord.

In the sensory spinal cord ganglion of the E20 rat embryo, all neurons stained for ChAT. However, the intensity of cytoplasmic staining of cells varied. The neuronal processes in the rat spinal cord ganglion that form the dorsal root of the spinal cord also contained ChAT. We found that the neuronal processes stained more intensely as compared to their bodies (Fig. 1a).

ChAT immunopositive structures of the gray matter of the cervical part of the spinal cord were located in the deep layers of the dorsal roots and Rexed's lam-

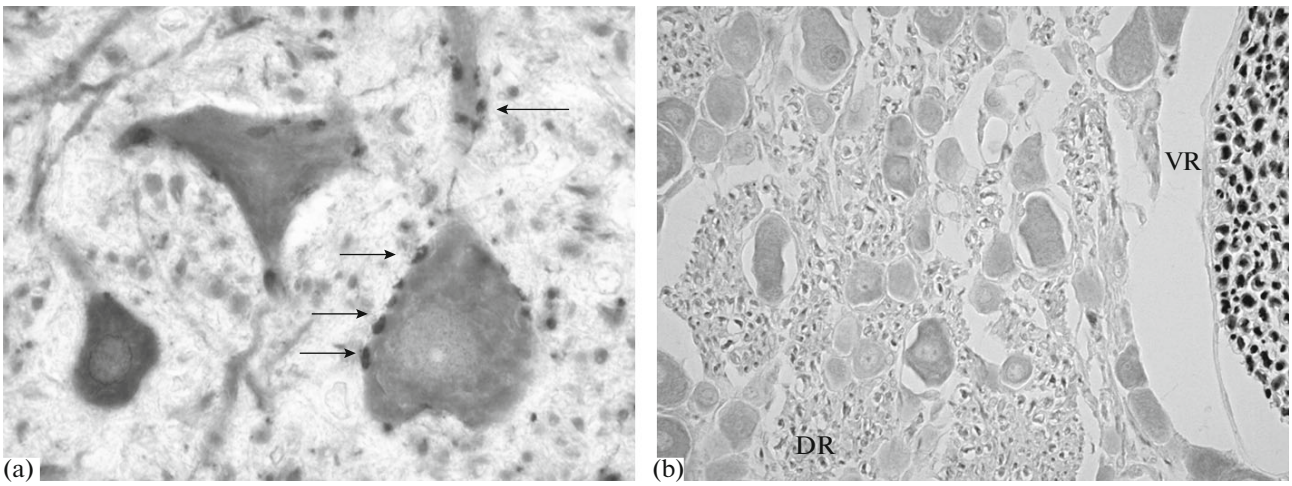

Fig. 2. Cholinergic neurons of (a) the ventral horn of the spinal cord and (b) sensory ganglion of an adult rat. Immunohistochemical staining for choline acetyltransferase. Arrows, cholinergic boutons; DR, fibers of the dorsal root of the spinal cord; VR, fibers of the ventral root of the spinal cord. Magnification: (a) ×1000; (b) ×400.

inae VI, IX, and X. In the dorsal horn gray matter (laminae III–V), we observed a few small neurons up to 16 × 7 μm in size with intensely stained cytoplasm. In the laminae I–V, we observed ChAT containing fibers. Larger immunopositive cells were located in the central gray matter of Rexed's lamina X. The cells were fusiform or round and were 18 × 14 μm in size. Their processes also contained ChAT and formed a network. In the spinal cord intermediate gray matter, ChAT-containing neurons were located on the border between Rexed's laminae VI and VII. We clearly observed large stellar immunopositive neurons up to 30 × 16 μm in size with ChAT containing thick processes. ChAT containing cells of the ventral horns were grouped in Rexed's lamina IX. These cells were large from 17 × 12 to 31 × 27 μm in size and had numerous immunopositive processes, which formed a dense network. The processes of motoneurons, which form the ventral root of the spinal cord, were also stained. In this region, the intensity of cytoplasmic staining of cells was lower as compared to the cells of the central gray matter and Rexed's lamina VI. We found ChAT-immunopositive structures, which were similar to axosomatic synapses, on some cellular bodies of lamina IX. Similar structures were observed on dendrites of neurons where they probably corresponded to axodendritic synapses. Cells of the intermediate gray matter exhibited the maximum level of ChAT staining.

Neurons of the sensory ganglion of newborn rats and their processes, which form the dorsal root of the spinal cord, stained weakly (Fig. 1b).

Studies on ChAT expression in the cervical part of the spinal cord of adult rats using light transmission or confocal laser microscopy revealed several areas with intense immunohistochemical staining. In the dorsal horns of the spinal cord, ChAT immunoreactivity was mostly observed in neuronal processes, which formed a network in Rexed's laminae I–VI. However, we also revealed several cells that express ChAT in laminae II–IV. We found that ChAT containing neurons of laminae II–III were fusiform and from 17 × 6–to 20 × 8 μm in size, whereas cells of Rexed's lamina IV were mostly round and 15–16 μm in diameter. In the central gray matter or Rexed's lamina X, ChAT expression was found in the cytoplasm of small interneurons that were 25 × 15 μm in size. These cells were fusiform or oval and extended in the mediolateral direction. The processes of these cells also contained ChAT and formed a network. Some immunopositive fibers projected to Rexed's lamina IX. On the border between laminae VI and VII, we observed ChAT immunopositive fusiform neurons that were 30 × 24 μm in size. The cells were oriented mediolaterally and some their processes reached Rexed's lamina X and the lateral horns of the spinal cord. In the gray matter of the ventral horns of the spinal cord i.e., in Rexed's lamina IX, we detected ChAT-containing large and medium neurons that were 44 × 34 and 35 × 27 μm in size, respectively, with stained cytoplasm. We also found weakly stained immunopositive neurons. Furthermore, in some cells of the ventral horns of the spinal cord, the enzyme was located in the nuclei whereas the nucleoli were immunonegative (Fig. 2a). In the gray matter of the ventral horns, a dense network of ChAT containing fibers was observed. ChAT was also expressed in neuronal processes that went through the white matter and formed the ventral roots of the spinal cord. We observed immunopositive structures that were similar to synaptic boutons on the plasma membranes of large and medium ChAT containing neurons (Fig. 2a). Similar structures were observed on ChAT immunonegative neurons of Rexed's laminae VIII–IX.

In adult rats, neurons of the sensory ganglion of the dorsal root of the spinal cord and their processes were ChAT immunopositive (Fig. 2b). Most of these neurons stained less intensely as compared to neurons of the spinal cord.

DISCUSSION

Changes in the distribution of ChAT in structures of the central and peripheral nervous system are understudied. Only single studies have examined cholinergic cells in the developing spinal cord [20–22]. In these studies, mouse monoclonal (3F12) or rabbit polyclonal antibodies were used for immunohistochemical detection of ChAT. Use of the well validated [13, 23, 24] polyclonal goat antibody (AB144) allowed us to reveal some ontogenetic features of the formation of ChAT-containing structures in the spinal cord and sensory ganglion.

Our study shows that at 20 days of the embryogenesis, four groups of developing cholinergic neurons could be observed in the cervical part of the spinal cord. These groups, which are specific for adult spinal cord, were small cells of the ventral horns, immature neurons of the central gray matter, developing neurons of the intermediate gray matter, and cells of the ventral horns.

Rat spinal cord on day 20 of the prenatal development is less differentiated and thus, it contains fewer immunopositive cells in Rexed's lamina X.

Here, we show that in interneurons of Rexed's laminae VI–VII, which modulate motor neurons [25, 26], the immunohistochemical reaction for ChAT was most expressed as compared to other groups of cholinergic cells of the spinal cord. It is possible that this immunoreactivity of neurons of the intermediate gray matter of the spinal cord is related to higher ChAT content.

In the present study, we found ChAT in the nuclei of sparse motor neurons of the ventral horns of the spinal cord of adult rats. However, the nucleoli of these cells remained immunonegative. Some authors suppose that intranuclear ChAT of human cells may activate transcription of the gene for the choline transporter CHT1 by binding of nuclear acetyl coenzyme A or acetylation of transcription proteins [27]. We believe that under some pathological functional conditions permeability of nuclear membrane is modified and thus, ChAT may come from the cytoplasm to the nucleus. However, this fact should be studied additionally. It is noteworthy that in newborn rats motor neurons exhibit lower immunoreactivity as compared to motor neurons of adult rats and neurons of other regions of the spinal cord of newborn rats. This fact cannot be correctly explained now from the physiological point of view. We found that cells of the sensory ganglion of rat embryo differed in their cytoplasmic staining. The fibers of nerve cells that form the ventral root of the spinal cord were stained more intensely, compared to the fibers of the dorsal roots. These differences in immunoreactivity were observed in rat spinal cord at all developmental stages that we studied and should be studied additionally.

ChAT immunohistochemistry allowed us to reveal large cholinergic structures in the spinal cord that were similar to C-boutons, which provided connections between the cholinergic interneurons of the gray matter and motor neurons [28]. The presence of the integral protein of synaptic vesicles synaptophysin in these structures [29] supports our conclusion that these structures are presynaptic boutons. These synapses were found not only near motor neurons in newborns and adult rats but also on developing immature motor neurons of the spinal cord in E20 embryos. Moreover, axosomatic and axodendritic immunopositive synapses in Rexed's lamina IX were stained more intensely than the cell bodies of motor neurons and interneurons of Rexed's lamina X, which form these synapses. This difference was observed using both transmission light and laser confocal microscopy. The results of confocal microscopy of the sections of the spinal cord of adult rat are presented in a diagram of the intensity of the fluorescence of the cytoplasms of motor neurons, cholinergic C-boutons, and the neuropil in Fig. 3. Higher intensity of fluorescence of the synaptic boutons was associated with an accumulation of ChAT, which was synthesized in the perikarya of cholinergic neurons of the central gray matter and transported by axoplasmic flow into the nerve terminals, where it was involved in the synthesis of the neurotransmitter. In E20 embryos, lower intensity of ChAT staining in presynaptic boutons as compared to newborn rats was probably due to the smaller sizes of the synapses and less intense staining in neurons of the central gray matter, which form these synapses [28].

In the present study, we additionally compared ChAT reactivity in neurons of the central and peripheral nervous system. In the modern literature, data on the capability of immunohistochemical detection of ChAT in neurons of the peripheral nervous system using the same antibody are controversial. Some studies indicate that most of the commercially available anti-ChAT antibodies may efficiently detect cholinergic structures in the brain or spinal cord only, whereas peripheral neurons and fibers are stained weakly [30]. As an example, parasympathetic postganglionic neurons are stained relatively poorly [30]. In 2000, a hypothesis was proposed on the difference between the chemical structures of ChAT expressed in peripheral tissues and ChAT expressed in the CNS [31]. The authors reported the presence of alternative splicing of ChAT mRNA, which resulted in the formation of ChAT isoenzyme with a molecular weight of 55 kDa, which was designated as ChAT of the peripheral type (pChAT). This ChAT is predominantly specific for peripheral neurons and neural fibers. The enzyme, which is expressed in neurons of the CNS and PNS,

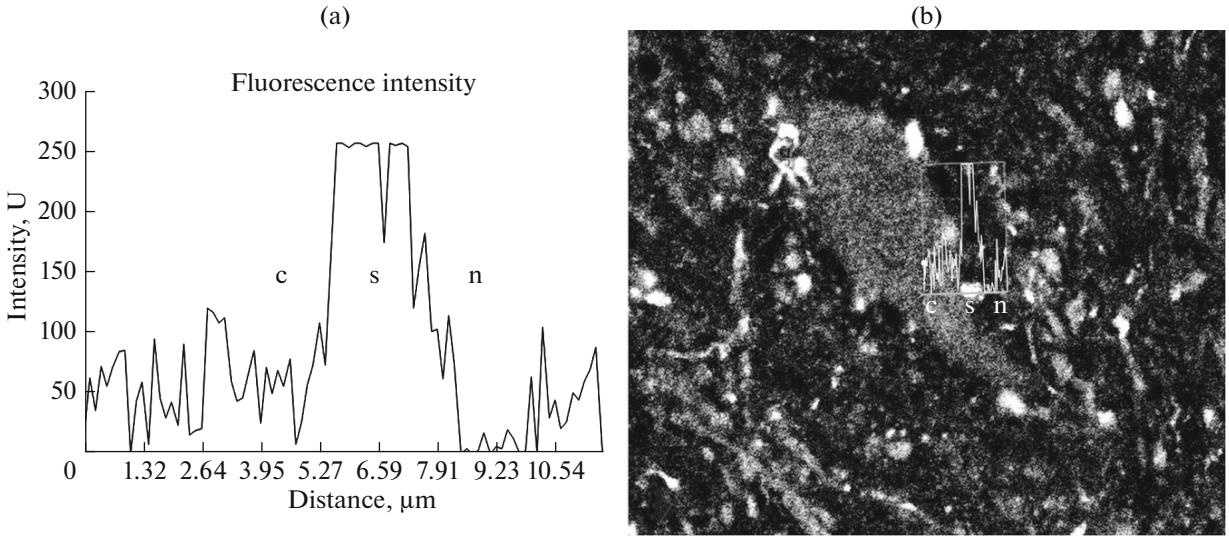

Fig. 3. ChAT fluorescence intensity. (a) Diagram of fluorescence intensity in the cytoplasm (c) of cholinergic neuron of an adult rat, synapse (s), and neuropil (n) within the 14-μm segment, which is indicated in microphotography (b). Immunohistochemical staining for choline acetyltransferase followed by visualization of the reaction product with a Cy5 fluorochrome. Confocal laser microscopy. C-Apochromat 63× lens with water immersion.

was indicated as ChAT of the general type (gtChAT) with a molecular weight of 68 kDa [31]. The anti-ChAT antibody that was used in the present study is capable of detecting the enzyme with a molecular weight of 68 kDa. It is not known how efficiently this antibody interacts with various pChAT epitopes. Data on the contents of different types of ChAT in sensory neurons are controversial. Thus, Bellier and Kimura [32] reported that neurons of the sensory ganglion do not express gtChAT, whereas other authors demonstrate the presence of gtChAT using Western blotting [12]. In the present study, we revealed the presence of ChAT in all neurons of the rat sensory ganglion at all stages of ontogeny although the cytoplasmic staining in these neurons was substantially lower as compared to CNS neurons. This fact may be explained by the presence of a specific type of the enzyme in cells of the sensory ganglion. This isoenzyme may be formed due to posttranslational modifications or alternative splicing and exhibit lower affinity to the antibody used. Posttranslational modifications and immunogenic epitopes of ChAT are not described completely; therefore, the absence of strong immunoreactivity with known anti-ChAT antibodies cannot form a sufficient basis for the exclusion of these neurons of the PNS from a population of cholinergic cells according to a formal sign. Our data begin a trend towards the study of the molecular mechanisms of the functional specialization of central and peripheral cholinergic neurons.

Thus, in the present study, we described ChAT-immunopositive structures in the spinal cord and sensory ganglion at various stages of ontogeny. We showed that at all stages of ontogeny that we studied, four groups of cholinergic neurons are present in the cervical part of the rat spinal cord: small cells of the dorsal horns, neurons of Rexed's lamina X, neurons of Rexed's laminae VI–VII, and cells of the ventral horns. We found that all neurons of the rat sensory ganglion express ChAT at all stages of ontogeny. Additionally, we observed cholinergic neurons with weak immunoreactivity to goat anti-ChAT antibody. These cells included developing interneurons of the central gray matter of the spinal cord of E20 rat embryos, some motor neurons of the spinal cord of newborn and adult rats, and cells of the sensory ganglion at all stages of ontogeny.

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