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APPEARANCE AND DISTRIBUTION OF ACETYLCHOLINESTERASE ACTIVITY
IN THE DEVELOPING NERVOUS TISSUES IN RAT EMBRYOS*

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INDEXING WORDS

acetylcholinesterase; histochemical study; nervous tissues; rat embryos

SYNOPSIS

Acetylcholinesterase (AChE) activity in the developing nervous tissues in rat embryos were histochemically investigated. Light microscopically in the 10.5- and 11.5-day-old embryos, AChE activity appeared to be negative in any embryonic tissue. In the 12.5-day-old embryo, a few cells in the mantle layer of the neural tube (NT), spinal ganglia (SPG) and sympathetic ganglia (SMG) displayed weak to moderate AChE activity. In the 13.5- and 14.5-day-old embryos, moderate to intense AChE activity was demonstrated in the mantle layer and posterior funiculus in the NT, SPG and SMG and in the spinal nerves. Electron microscopically, in the 11.5-day-old embryo, AChE activity was first discernible in some NT cells, and thereafter, the cells exhibiting AChE activity rapidly increased in the NT, SPG and SMG. Reaction products for AChE were well localized in the cisterns of the nuclear envelope and endoplasmic reticulum of the related cells.

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These findings strongly suggest a close relationship between the increase of AChE activity in the developing nervous tissues and the formation of a functional network among them. We speculate that AChE may be involved in regulation of cell to cell contacts, including the formation of synaptic contacts in the developing nervous tissues.

INTRODUCTION

During the early embryonic development, acetylcholinesterase (AChE) and nonspecific cholinesterase (ChE) are demonstrable not only in the nervous tissues but also in various kinds of tissues in the chick embryo^{4, 14, 15, 16, 17, 18}, rat embryo^{8, 28} and mouse embryo²⁷. Drews⁶ named these enzymes "embryonic cholinesterases" and suggested that they may play an important role in regulation of cellular movement during morphogenesis.

Previously, using ³H-thymidine autoradiography and AChE histochemistry on the same sections, we studied the appearance and distribution of AChE activity in the neural tube cells of the chick embryo, and reported that AChE activity rapidly increased in intensity soon after the final cell division, and also that increase of AChE activity can be used as the first sign of cellular differentiation of neurons^{16, 17}. To date, however, the significance of these cholinesterases in the embryonic tissues has not yet fully been elucidated.

In the present study, to investigate the possible role of AChE in the developing nervous tissues, appearance and distribution of AChE activity in the neural tube, spinal ganglia and sympathetic ganglia of rat embryos were histochemically examined by light and electron microscopy.

MATERIALS AND METHODS

Animals

Wistar rats were kept at 21 ± 2 C with light-dark changes every 12 hr. Adult rats at least 3.5 months old were mated overnight, and following morning, presence of sperm was examined in a vaginal smear. It was assumed that rats with a positive smear had mated within 2 hr of midnight (24:00), and the conceptuses were thus

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presumed to be 0.5 days old at noon (12:00)²⁾. Ten pregnant rats between 10.5 and 14.5 days of gestation (2 rats on each pregnant day), were decapitated under light ether anaesthesia, uteri were removed and embryos were immediately dissected from them in Hank's balanced salt solution.

Light microscopic histochemistry

These embryos were fixed for 12 hr at 4 C in 4% paraformaldehyde diluted with 0.1 M cacodylate buffer (pH 7.3) containing 5% sucrose and rinsed overnight at 4 C in the same buffer. Transverse sections about 200 μ m thick were cut with a Vibratome, frozen two or three times in dry ice-cooled acetone and incubated for 2 hr at 37 C in the medium of Karnovsky and Roots⁷⁾ containing acetylthiocholine iodide as substrate and 10^{-5} M iso-OMPA (tetraisopropyl pyrophosphoramidate) as an inhibitor of non-specific cholinesterase. After washing but without postfixation in osmium tetroxide solution, they were dehydrated in graded alcohols and embedded in an epoxy resin mixture (Quetol 812, Nisshin E.M., Tokyo). Plastic sections about 2 μ m thick were cut with glass knives and examined under an ordinary light microscope.

Electron microscopic histochemistry

These embryos were fixed for 3 hr at 4 C in a mixture of 2% glutaraldehyde and 2% paraformaldehyde dissolved in 0.1 M cacodylate buffer (pH 7.3) containing 5% sucrose and 2 mM calcium acetate, and rinsed overnight at 4 C in an isotonic buffer (which consisted of 1.664% sodium sulfate solution 74 ml, 0.2 M sodium cacodylate 25 ml and 0.2 M calcium acetate 1 ml). Non-frozen sections about 200 μ m thick were cut with the Vibratome and incubated for 2 hr at room temperature in a medium containing acetylthiocholine iodide and 10^{-5} M iso-OMPA, according to the method described by Lewis and Shute^{12, 13)}. After washing for 1 hr in the isotonic buffer, these sections were treated with a sodium sulfide solution for 1 hr at 4 C, postfixed for 1 hr at 4 C in Dalton's solution⁵⁾, dehydrated and embedded in the epoxy resin mixture. Ultrathin sections were prepared with glass knives, stained lightly with uranyl acetate and examined in an electron microscope, JEM 100-SX (JEOL, Tokyo, Japan).

Histochemical controls

Both for light and electron microscopy, histochemical controls were performed as follows: (1) incubation in a substrate-free medium; (2) incubation in a medium containing 10^{-4} M BW284C51 (1,5-bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one-dibromide), a specific inhibitor for AChE.

RESULTS

In the present study, appearance and distribution of acetylcholinesterase (AChE) activity was examined in transverse sections at the lower rhombencephalic and upper cervical levels of rat embryos between days 10.5 and 14.5 of gestation.

10.5-day-old embryo

The neural tube (NT) in the cervical region and the neural plate (NP) in the rhombencephalic region consisted of a pseudostratified columnar epithelium with 3 to 4 layers of nuclei. The majority of cells in these tissues were spindle-shaped, and had a large oval nucleus and a scanty cytoplasm. A few round cells being in mitosis were observed just beneath the lumen. Between the NT and somite and between the NP and surface ectoderm, several pleomorphic mesenchymal cells were distributed. By electron microscopy, ultrastructure of the cells constituting the NT and NP, and mesenchymal cells was essentially similar. The nucleus showed low electron density and had one or two prominent nucleoli. The cytoplasm was filled with polysomes, and contained a few short sections of rough-surfaced endoplasmic reticulum (RER), a few poorly-developed Golgi complexes and some small round or ovoid mitochondria. On the basis of the morphological appearance, the cells in the NT and NP were regarded as undifferentiated neuroepithelial cells, and formed a matrix.

AChE activity was not discerned in any embryonic tissue at light or electron microscopic level.

11.5-day-old embryo

The wall of the NT consisted exclusively of matrix with 4 to 5 layers of nuclei. Anlages of spinal ganglia (SPG) or sympathetic

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ganglia (SMG) were not recognized. Ultrastructure of the cells in the NT and the mesenchymal cells was similar to that on the previous day.

By light microscopy, AChE activity was hardly discerned anywhere in the embryonic tissues. Only by electron microscopy, some neuroepithelial cells exhibiting weak enzymatic activity in cisterns of the nuclear envelope and of a few short sections of RER were noted (Fig. 1).

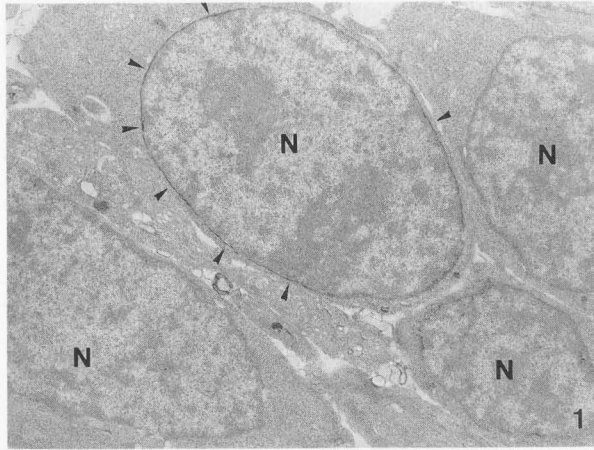


Fig. 1. Electron micrograph of neuroepithelial cells in an 11.5-day-old rat embryo. Some cells show very weak AChE activity in the cisterns of the nuclear envelope (arrowheads). N: nucleus. x 6000

12.5-day-old embryo

The wall of the NT consisted mainly of the matrix with 4 to 5 layers of nuclei. Some round cells formed a thin mantle layer in the ventrolateral and dorsolateral regions of the NT. Ventral spinal root fibers were recognized. SPG were first identified lateral to the NT, and SMG, dorso-lateral to the dorsal aorta. The cells constituting the former were spindle-shaped, and had a large ovoid nucleus, and those constituting the latter were polygonar and had a round nucleus. Ultrastructure of the neuroepithelial cells was similar to that in the 10.5- and 11.5-day-old embryos. The cells in the mantle layer of the NT had a round electron-light nucleus, and a relatively abundant cytoplasm. The cytoplasm contained several sections of RER, and several ovoid or elongated mitochondria. These cells were considered to be young

neurons. The cytoplasm of the cells in the SPG and SMG had the same ultrastructural appearance of the young neurons in the NT.

Light microscopically, it appeared that the majority of the cells in the NT were negative in AChE activity. A few round cells seen in the mantle layer in the ventro-lateral region of the NT displayed very weak AChE activity along the nuclear membrane and in the cytoplasm (Fig. 2). In the SPG and SMG, a few cells exhibiting weak to moderate AChE activity along the nuclear membrane and in the cytoplasm were noted (Fig. 3). Electron microscopically, enzymatic activity was detected in the cisterns of the nuclear envelope and some cisterns of RER in the cells constituting the NT, SPG and SMG (Fig.4). Besides these tissues, weak to moderate AChE activity was demonstrated in the myotome.

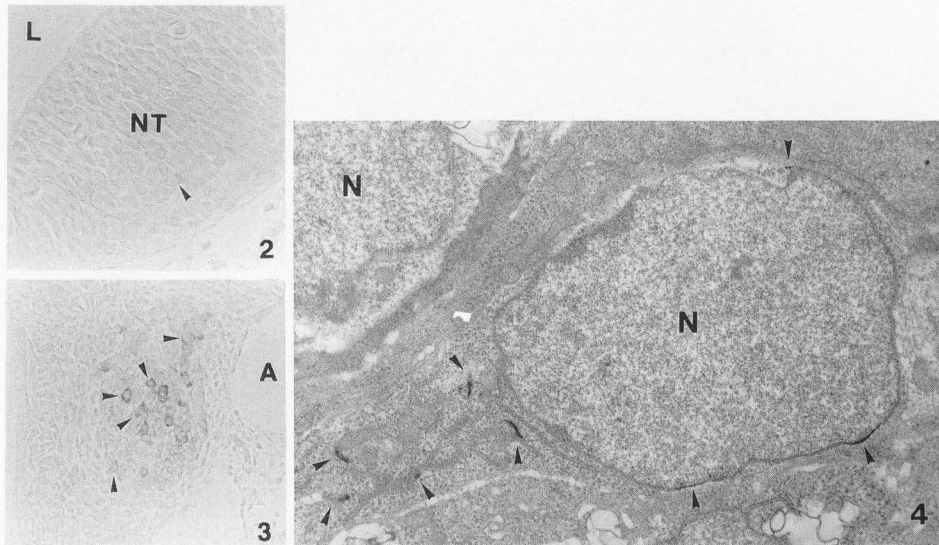


Fig. 2. Transverse section of a 12.5-day-old embryo at the rhombencephalic level. Almost all cells constituting the neural tube wall appear to be negative in AChE activity. Only a few cells in the mantle layer show very weak AChE activity (arrowhead). NT: neural tube, L: lumen of the neural tube. x 280

Fig. 3. Transverse section of a 12.5-day-old embryo at the upper cervical level. Some cells in the sympathetic ganglia exhibit weak to moderate AChE activity (arrow-heads). A: dorsal aorta. x 220

Fig. 4. A young neuron seen in the mantle layer of the cervical cord of a 12.5-day-old rat embryo. Weak AChE activity is demonstrated in the cisterns of the nuclear envelope and some short sections of RER (arrowheads). N: nucleus. x 7,000

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13.5 and 14.5-day-old embryo

The wall of the NT consisted of the matrix with 5 to 6 layers of nuclei and the thick mantle layer. The mantle layer in the ventro-lateral region was evidently thicker than that in the dorso-lateral region. The thick bundles of the spinal nerve fibers were demonstrated. The number of cells in the SPG and SMG rapidly increased.

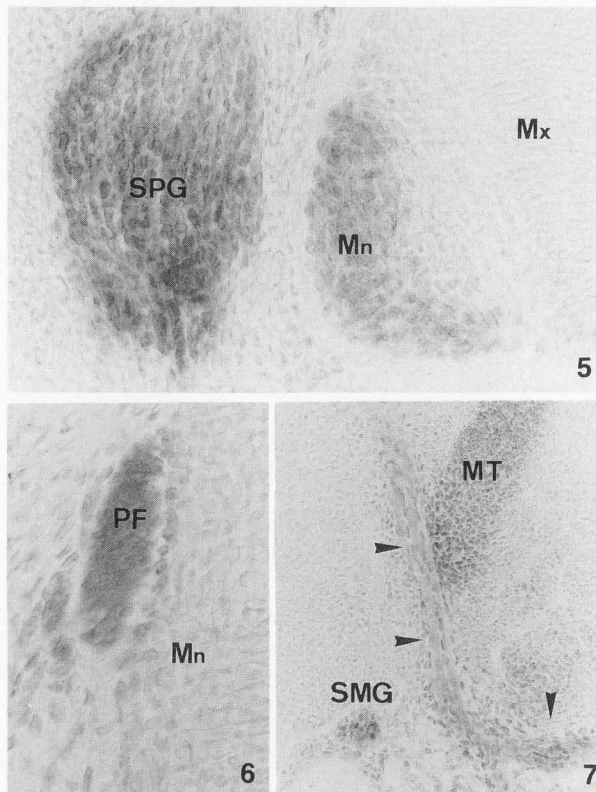


Fig. 5. Transverse section of a 13.5-day-old rat embryo at the cervical level. Moderate AChE activity is demonstrated in the lateral half of the mantle layer (Mn) of the neural tube, and intense ChE activity in the spinal ganglion (SPG). Mx: matrix of the neural tube. x 280

Fig. 6. Dorsal part of the neural tube of a 13.5-day-old embryo. Posterior funiculus (PF) shows intense AChE activity. Mn: mantle layer of the neural tube. x 300

Fig. 7. Transverse section at the cervical level of a 13.5-day-old embryo. Myotome (MT) and sympathetic ganglion (SMG) exhibit moderate to intense AChE activity, and spinal nerve (arrowheads), weak to moderate activity. x 120

Moderate to intense AChE activity was demonstrated in the mantle layer and the anlage of posterior funiculus in the NT, SPG and SMG. Spinal nerves displayed moderate enzymatic activity, and myotomes, intense activity (Figs. 5,6,7). Electron microscopically, numerous cells in the NT, SPG and SMG exhibited moderate AChE activity in the cisterns of the nuclear envelope and sections of RER (Fig. 8).

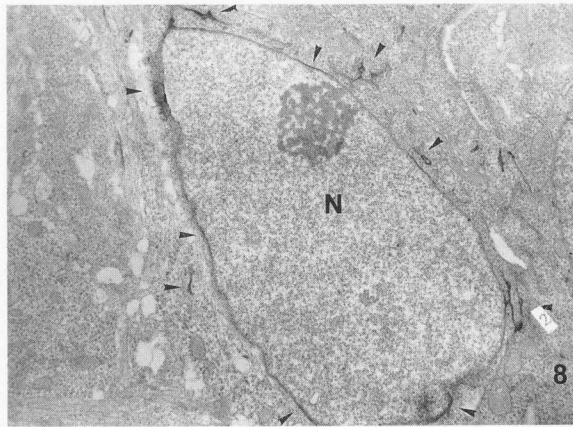


Fig. 8. A young neuron in the mantle layer of the neural tube of a 13.5-day-old embryo. Moderate AChE activity is demonstrated in the cisterns of the nuclear envelope and several sections of RER (arrowheads). N: nucleus. x 4,300

Histochemical controls

In the sections incubated in the substrate-free medium, no reaction products were detected in any embryonic tissue. The reaction was strongly inhibited when the sections were incubated in the medium containing 10^{-4} M BW284C51.

DISCUSSION

In the present study, a few cells displaying weak to moderate acetylcholinesterase (AChE) activity were light microscopically first demonstrated in the neural tube (NT), spinal ganglia (SPG) and sympathetic ganglia (SMG) of the 12.5-day-old rat embryo. On days 13.5 and 14.5 of gestation, the enzymatic activity in these tissues became strong. Sarrat²⁵⁾ and Schoenen²⁶⁾ reported that AChE activity was histochemically first detected in the rat spinal cord and SPG on day 15 of gestation. In these studies, AChE histochemistry was performed on frozen sections. In our study, 100 μ m thick sections cut with a Vibratome were

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incubated in the medium of Karnovsky and Roots⁷⁾ for AChE reaction and embedded in an epoxy resin mixture. Two- μ m thick plastic sections were prepared from them and used for light microscopy. We found that our method is much more suitable for precise observations than the method using frozen sections, especially from the young embryonic tissues^{14, 15, 17, 18)}. Biochemically, Burt³⁾ reported that in the rat lumbar cord, AChE activity rapidly increased from 13th day of gestation. Our histochemical observations are in good accordance with the biochemical data presented by Burt³⁾.

Recently, we histochemically investigated the distribution of lactate dehydrogenase (LDH), succinate dehydrogenase (SDH) and cytochrome c oxidase (Cytox) activity in postimplantation rat embryos, and found that the levels of SDH and Cytox activity in the mantle layer of the NT, SPG and myotome in the 12.5-day-old embryo were evidently higher than those on the previous day¹⁹⁾. These findings indicate that in these cells, the tricarboxylic acid cycle and electron-transport system begin to become more active between days 11.5 and 12.5 of gestation, and suggest that these cells begin to differentiate in the respect of energy-yielding metabolism at these stages. In the 11.5-day-old embryo, the cells constituting the NT and neural plate (NP) were morphologically undifferentiated neuroepithelial cells. Only by electron microscopy, some neuroepithelial cells displaying weak AChE activity were discerned. In the 12.5-day-old embryo, several round cells, which were morphologically regarded as young neurons, formed a distinct mantle layer. While by light microscopy, only a few neurons exhibited weak AChE activity, and many of the neurons appeared to remain still negative. Using ³H-thymidine autoradiography and AChE histochemistry on the same sections, we examined the relationship between the cellular differentiation and distribution of AChE activity in the NT cells of the chick embryo, and reported that AChE activity rapidly increased in intensity soon after the final cell division^{16,17)}. Using ³H-thymidine autoradiography, Altman and Bayer¹⁾ reported that in the rat embryo, the first neurons appeared in the upper cervical cord on day 11 of gestation. Judging from these facts, the result from the present study suggests that in the rat embryo, 1 to 1.5 days elapse between the increase of AChE and the initiation of cellular

differentiation in the NT cells. This may be due to the difference in species. As to the intracellular localization of AChE activity, there was no difference between tissues or species^{14, 15, 16, 21, 27, 28)}.

AChE, a catabolic enzyme of acetylcholine, is one of the essential elements of cholinergic system. However, significance of AChE in the developing nervous tissues is not yet fully understood. Several authors reported that AChE and non-specific ChE were transiently demonstrated in the developing central nervous system and suggested that these transient cholinesterases may play a role in the development of neuronal connection^{9, 10, 22, 23, 24)}. In the chick embryo, increase of AChE in myotomes begins almost in parallele with that in the NT^{11,15)} and clearly before motor axons have arrived¹¹⁾. In our materials, AChE activity began to increase in the mantle layer of the NT, SPG, SMG and myotome between days 12.5 and 13.5 of gestation. On day 13.5, the spinal nerves which appeared to reach the myotome, and the posterior funiculus which contains nerve fibers from the SPG cells²⁹⁾ displayed moderate to intense AChE activity. These findings strongly suggest that there is a close relationship between increase of AChE activity and formation of a functional network, such as innervation of motor neurons and formation of a reflex arch, among these structures.

During the stages of major organogenesis, AChE is demonstrated not only in the nervous tissues, but also in the other tissues, such as surface ectoderm, somite, notochord in chick embryos^{6, 14)}, genital tract²⁷⁾, myocardium⁸⁾ and mandibular processes²⁸⁾ of mice and rats. Drews⁶⁾ named these cholinesterases "embryonic cholinesterases" and suggested that ChE at the early developmental stages is involved in cellular movement. We speculate that AChE may generally play an important role in regulation of cell to cell contact during histogenesis, including the formation of synaptic contacts in the developing nervous tissues, as suggested by Robertson et al.²³⁾ and Moody and Stein²⁰⁾.

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