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Differentiation of Cholinergic Neurons in Rat Spinal Cord under Conditions of Allotransplantation into a Peripheral Nerve and *In Situ* Development

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The method of ectopic transplantation of embryonic anlages of CNS allows studying histoblastic potencies of progenitor cells developing under conditions of changed microenvironment. Some progenitor cells in the transplants of rat embryonic spinal cord retained their ability to express choline acetyltransferase after transplantation into the sciatic nerve of adult animals. Comparative analysis of cholinergic neurons in the neurotransplants and neurons formed in rat spinal cord during normal ontogeny showed that choline acetyltransferase-positive cells after transplantation into the nerve reached morphological differentiation of motor neurons at later terms than cells developing *in situ*. They were scattered one by one and did not form nuclear nerve centers. We did not find structures similar to presynaptic cholinergic buds typical of intact spinal cord near these cells throughout the observation period. Solitary cholinergic neurons survived in the transplants for 19 months.

Key Words: *nerve; embryonic spinal cord; cholinergic neurons; choline acetyltransferase; immunohistochemistry*

Differentiation of embryonic neural stem/progenitor cell (NBTS) attracted much recent attention due to their active use in experiments aimed at the search of new ways of stimulation of reparative processes in CNS organs by using modern cell technologies [1,2,6,10]. As for the peripheral nervous system, there is evidence that embryonic CNS anlages and NBTS after transplantation into the injured nerve or the conduit connecting the proximal and distal segments of the transected nerve stimulate regeneration of the nerve in the recipient [11,14,17,18]. The fate of transplanted cells is unclear. Their differentiation into neuronal direction is primarily studied by using specific panneuronal marker proteins (β-tubulin, NeuN, neurospecific enolase, *etc*.) The transmitter characteristics of differentiating neurons remain an open question.

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Here we studied differentiation of cholinergic neurons developing in transplants of rat embryonic spinal cord (SC) in the peripheral nerve and compared it with the development of their analogs *in situ* during normal ontogeny.

MATERIALS AND METHODS

Experiments were performed on Wistar rats (n=50) of different ages. All manipulations were performed with strict adherence to Rules for Conducting Studies with Experimental Animals (Order No. 755 of the Ministry of Health of USSR, August 12, 1977). Cervical SC fragments were isolated from 14-day-old rat embryos. Embryonic anlages were transplanted to an adult rat under the perineurium of a sciatic nerve trunk with a glass cannula as described previously [9]. Sciatic nerves were preliminary crushed with a forceps for 40 sec. The mice were kept under standard vivarium conditions. Immunohistochemical analysis of nerve segments containing the transplants

was performed in 3, 7, 14, 60 days and 19 months after transplantation. For comparative study, fragments of cervical SC were isolated from17-day-old embryos (control for 3-day neurotransplants), newborn rats (control for 7-day transplants), and 7- and 53-day-old rats (controls for 2-week and 2-month transplants, respectively). Long-living transplants were compared with SC of old rats (19 months). Immunohistochemical detection of choline acetyltransferase (ChAT) was performed on paraffin sections [4,5]. Cholinergic neurons were detected using polyclonal goat anti-ChAT antibodies diluted 1:250 (E0466; Dako). For detection of the antigen–antibody complex by light microscopy, secondary biotinylated antigoat antibodies (dilution 1: 200, E0466; Dako) and streptavidin conjugated with peroxidase (Str/HRP; SpringBio) were used. The product of immunocytochemical reaction was visualized using DAB+ chromogen (Dako). Some sections were poststained with hematoxylin and eosin. The preparations were examined under a Leica DM750 microscope and photographed with Leica ICC50 camera.

RESULTS

Embryonic anlages of SC from 14-day embryos served as the initial material for transplantation. At this term, SC in rats consists of neuroepithelial cells and postmitotic neuroblasts located in the presumptive anterior horns (Fig. 1). Some cells in the anterior horn contained ChAT (Fig. 1, *c*). These were bipolar cells reaching 6×9 mm in size.

In 3 days after transplantation, the fragments of embryonic SC were easily identified in the center or at the periphery of the nerve trunk and were surrounded by thick ChAT-immunopositive nerve fibers of the recipient. The transplants consisted of a large number of progenitor cell, but none of them contained ChAT, which distinguished then from SC developing *in situ.* In 17-day-old rat embryos, cholinergic cells were found in the presumptive anterior horns of SC (Fig. 2, *a*). Moreover, they were found in the region surrounding the central channel from the bottom plate to the border groove. Elongated immunopositive cells with intensively stained processes were seen along the border of the ependymal zone in developing anterior horn of SC.

In 7 days after surgery, the neurotransplants surrounded by thick ChAT-immunopositive nerve fibers of the recipient were seen in the depth of nerve trunks. At this term, ChAT-positive cells were first detected in the transplants (Fig. 3, *a*, *b*). These small cells (10-15 μ) with poorly developed processes that did not rich the degree of structural differentiation observed in ChAT-containing cells developing *in situ* at the corresponding period (*i.e.* in neonatal rats).

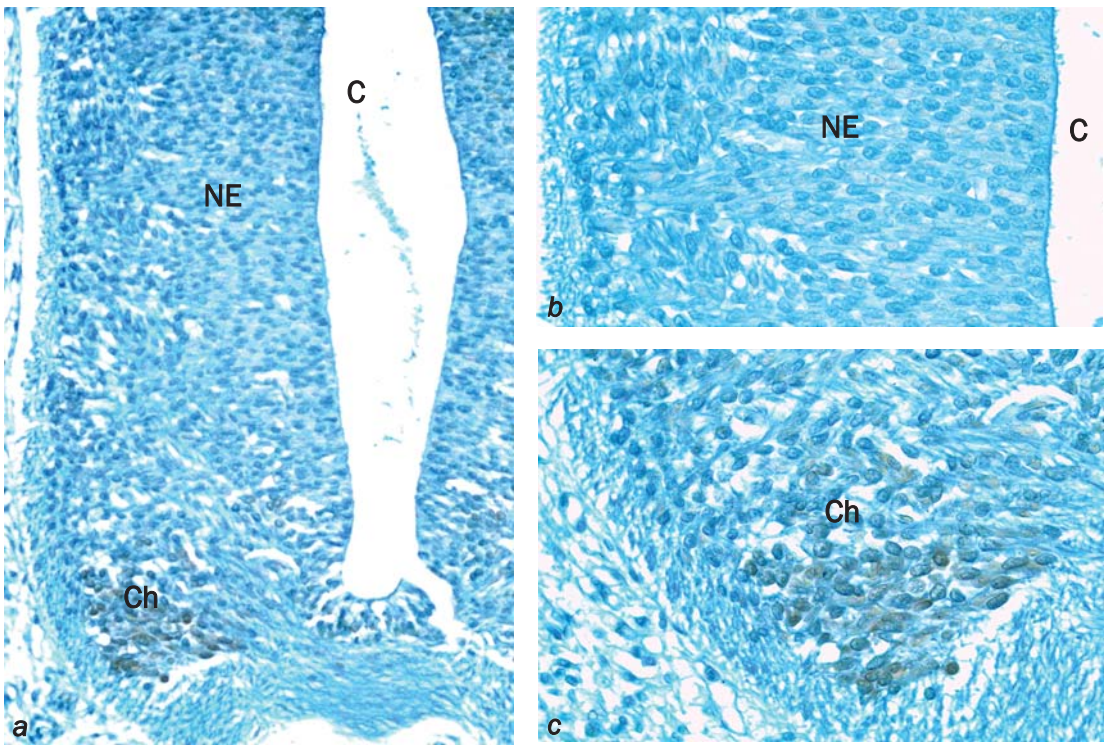

Fig. 1. Cervical portion of SC from a 14-day rat embryo. Immunohistochemical reaction for ChAT, astra blue staining, ×100 (*a*), ×400 (*b*, *c*). *a*) Total preparation; *b*, *c*) fragments of SC. NE: neuroepithelial cells; C: central channel; Ch: ChAT-positive cells in presumptive anterior horns.

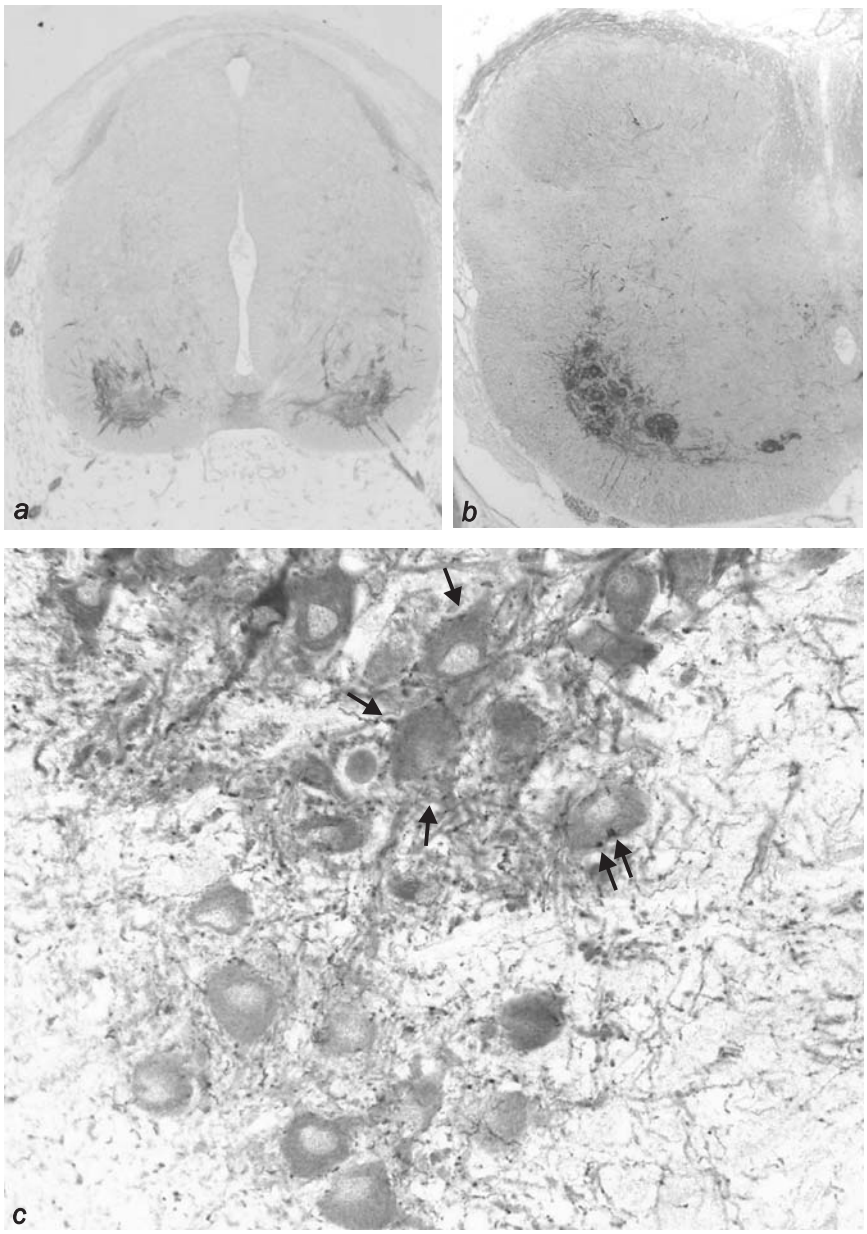

Fig. 2. ChAT-positive cells in rat SC during the embryonic and early postnatal period. Immunohistochemical reaction for ChAT, ×100 (*a*, *b*), ×400 (*c*). *a*) SC of a 17-day embryo; *b*, *c*) SC of a newborn rat. Arrows show ChAT-positive presynaptic buds.

In the cervical portion of SC of newborn rats, numerous triangular, round, or spindle-shaped ChAT-immunopositive cells of various size with intensively stained processes were seen (Fig. 2, *b*, *c*). Their distribution in SC of newborn rats was similar to distribution of cholinergic neurons in adult SC. The cells expressing the protein were located in the anterior horns (Rexed lamina IX), in the central gray matter (lamina X), and in the intermediate zone (laminae VI-VII); solitary cholinergic neurons were found in the posterior horns of SC. In neonatal rats, immunohistochemical staining for ChAT revealed presynaptic buds near formed motoneurons in the anterior horns (Fig. 2, *c*). They were seen as small round or clublike structures and demonstrated more intense ChAT-positive staining than perikarya of neurons and neuroblasts.

In 14 days, the number of ChAT-immunopositive cells in the transplants increased. They attained 10×20-15×13 μ in size and have long, sometimes branched processes. By staining intensity, size, and shape, they resembled motoneurons of the cervical SC of 7-day-old rats, but their density was much lower than in the control.

In 60 days, ChAT were detected in the perikarya and processes of nervous cells in the transplants (Fig. 4). By this term, neuropil was formed in the

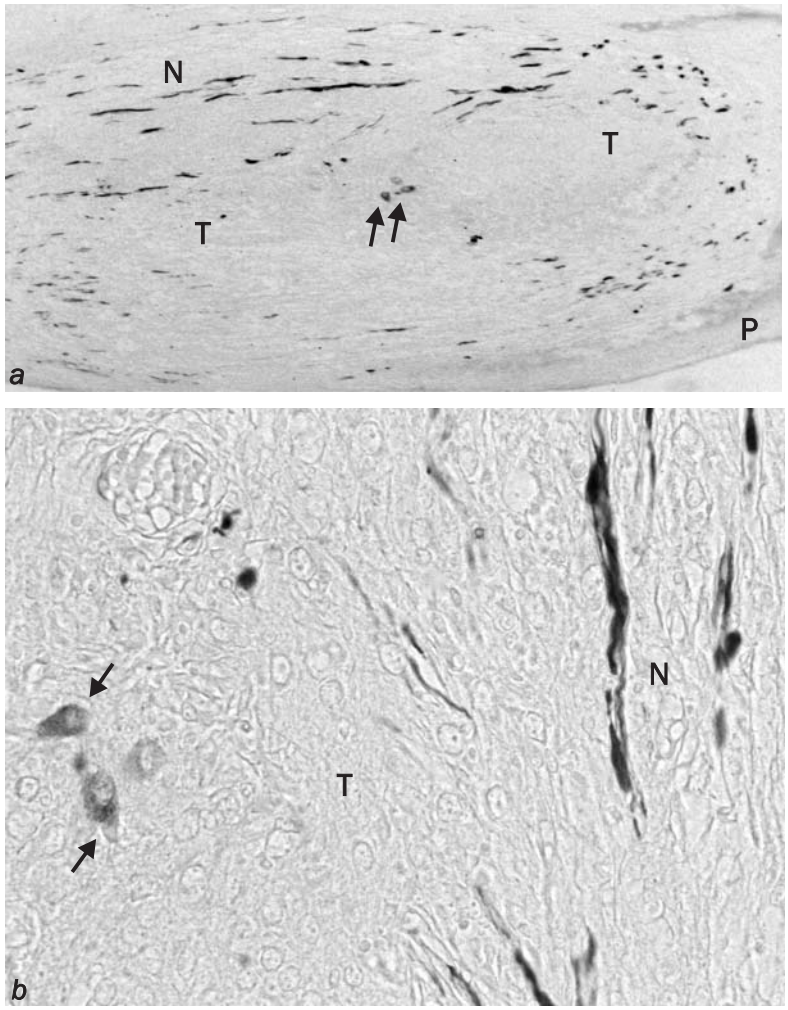

Fig. 3. Cholinergic neurons in transplants of rat embryonic SC in 7 days after transplantation into the nerve. Immunohistochemical reaction for ChAT, ×100 (*a*), ×400 (*b*). *a*) General view of the transplant; *b*) neurons in the transplant. Here and in Fig. 4: T: transplant; N: ChAT-positive fibers of the recipient; P: nerineurium of the nerve trunk. Arrows show ChAT-positive neurons of the transplant.

transplants. Some axons also contained ChAT, but did not form dense network normally observed in SC of rats of the corresponding age. Cholinergic axosomatic synapses were normally present in the anterior horns of rat SC. In neurotransplants, these structures were not found near the nerve cells. Moreover, degenerative changes were found in some ChAT-positive nerve cells. The cells were wrinkled with deformed nuclei and twisted processes (Fig. 4, *c*).

In 19 months, some neurons in the transplants still synthesized ChAT; they had abundant cytoplasm and long processes (Fig. 5, *a*). It should be noted that the intensity of staining for ChAT in old rats was lower than in newborn and 53-day-old animals. At the same time, some ChAT-immunopositive intensely stained synaptic buds were still seen near these neurons (Fig. 5, *b*). By the intensity of immunohistochemical reaction, neurons in the transplants were superior to motoneurons of old rats (Fig. 5, *a*, *b*). High intensity of immunohistochemical reaction for ChAT in neurons of long-living transplants can be related to compensatory response of cells developing in unusual microenvironment. This can also indicate changes in functional activity of these cells and the absence of ChAT transport to the peripheral target tissues. Low number of cholinergic neurons after transplantation can be explained by disruption of cell–cell interactions after transplantation, disturbances in progenitor cell migration during the early postoperative period, death of some precursors, and the absence of afferent and efferent relationships characteristic of SC.

Neurotransplantation of the fragments of embryonic CNS anlages is a convenient model for studies of histogenetic processes (proliferation, migration, and differentiation) in mammalian ontogeny [7,8]. Synthesis of neurotransmitters is an important differentiation criterion of nerve cells. The question whether neurons can synthesize typical transmitters after transplanta-

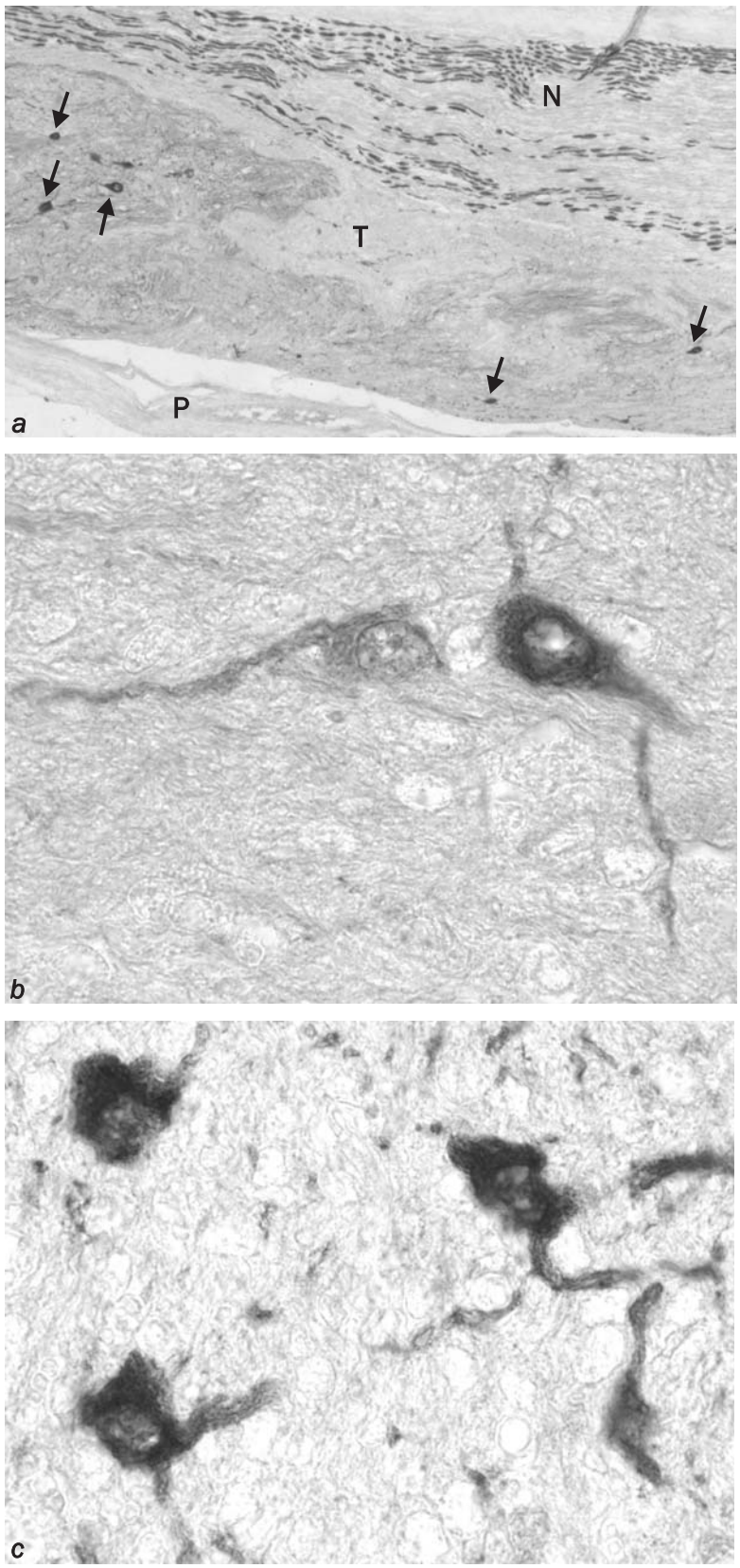

Fig. 4. Cholinergic neurons in transplants of rat embryonic SC in 60 days after transplantation into the nerve. Immunohistochemical reaction for ChAT, ×100 (*a*), ×1000 (*b*, *c*). *a*) General view of the transplant; *b*) neurons in the transplant; *c*) degenerative neurons in the transplant.

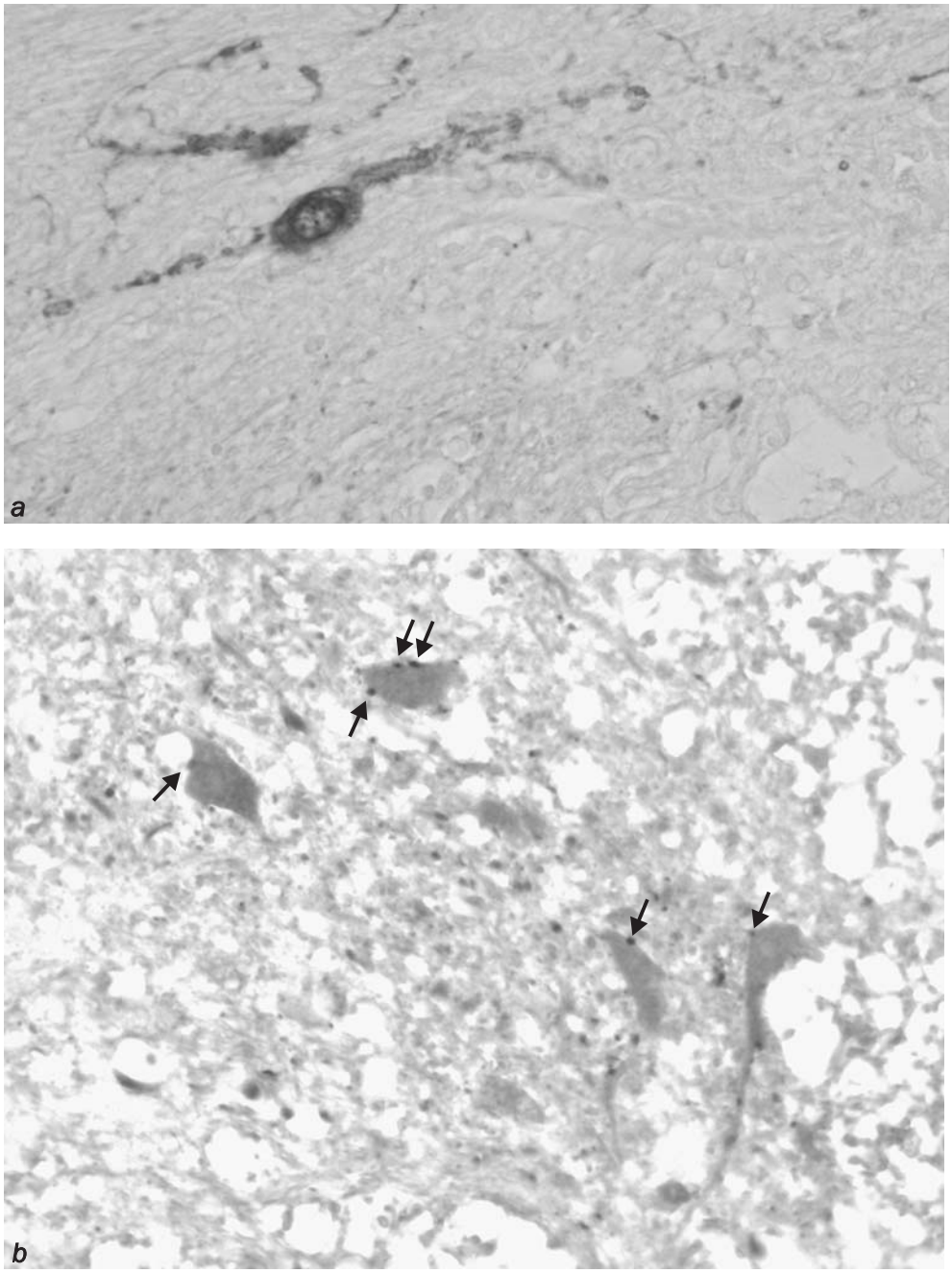

Fig. 5. Cholinergic neurons in long-living transplants of rat SC in 19 months after transplantation (*a*) and motoneurons of a 19-month-old rat. Immunohistochemical staining for ChAT, ×400 (*a*, *b*). Arrows show ChAT-positive neurons in the transplant.

tion is still debated. We found only few papers on transmitter synthesis in transplants developing in the peripheral nerves [8,11,13]. In our study, some cells of the embryonic SC realize their histoblastic potencies after transplantation into the peripheral nerve and differentiate into cholinergic neurons. Analysis of the development of embryonic SC fragments in the peripheral nerve revealed some features of the formation of ChAT-positive neurons developing in the transplant in comparison with normal development *in situ*. There is evidence that first ChAT-containing cells appear in SC embryogenesis very early, at the stage of presumptive anterior horns [15,16]. In our study, they formed small clusters in the anterior horns by day 14. Their number, density, and staining intensity increased until birth. In newborn rats, the distribution of ChAT-positive neurons is not confined to anterior horns and become similar to that in adult rat SC. At the early stages of transplant development, differentiation of transplanted progenitor cells was disturbed due

to surgical intervention and transfer of the embryonic anlage into new microenvironment: no ChAT-positive cells were detected in the transplants in 3 days after transplantation. Their absence can be explained by, first, suppression of ChAT synthesis in transplanted ChAT-immunopositive cells in response to damage, and second, death of some transplanted cells during the early postoperative period. Apoptotic death of cells in the transplants of embryonic CNS anlages at the early terms after transplantation has been previously demonstrated [7]. Interestingly, more differentiated progenitor cells more often undergo degeneration in the neurotransplants [12].

Thus, we revealed structures similar to presynaptic buds of axosomatic synapses near the neural elements in the anterior horns of SC in newborn rats. These structures contain synaptophysin, an integral protein of synaptic vesicles, which confirms their synaptic nature [3]. In neurotransplants, similar structures were not detected near the cholinergic neurons throughout the observation period.

The decrease in the number of ChAT-immunopositive neurons in the transplants in 19 months after transplantation can be explained by processes typical of long-living nerve transplants. Pronounced destructive changes in blood vessels and degeneration of the nerve and glial cells were previously demonstrated in these transplants [7].

Thus, our findings suggest that some neurons formed in the transplants of rat SC and developing in the nerve are cholinergic, *i.e.* progenitor cells from embryonic SC retain their characteristic phenotype and the ability to synthesize ChAT in changed microenvironment. Comparative analysis of cholinergic neurons in the neurotransplants and neurons formed in rat SC during normal ontogeny showed that ChAT-positive cells after transplantation into the nerve reached morphological differentiation of motor neurons at later terms than cells developing *in situ*. In the transplant, these cells were scattered singly, *i.e.* did not form nuclear nerve centers. No structures resembling presynaptic cholinergic buds typical of intact SC were found near these cells throughout the observation period. Solitary cholinergic neurons survived in the transplants over 19 months.

These findings can be useful for understanding of the fundamental mechanisms of histogenesis and regeneration in the nervous system.

REFERENCES

1. M. A. Aleksandrova, A. V. Revishchin, O. V. Podgornyi, *et al*., *Bull. Exp. Biol. Med*., **137**, No. 3, 262-265 (2004).
2. M. A. Aleksandrova, G. T. Sukhikh, R. K. Chailakhyan, *et al*., *Bull. Exp. Biol. Med*., **141**, No. 1, 152-160 (2006).
3. E. A. Kolos and D. E. Korzhevskii, *Morfologiya*, **144**, No. 4, 76-79 (2013).
4. D. E. Korzhevskii, I. P. Grigor'ev, O. V. Kirik, *et al*., *Morfologiya*, **144**, No. 6, 69-72 (2013).
5. D. E. Korzhevskii, I. P. Grigor'ev, O. V. Kirik, *et al*., *Zh. Evoluts. Biokhim. Fiziol*., **50**, No. 2, 157-160 (2014).
6. E. V. Loseva, *Neurokomp'utery: Razrabotka Priminenie*, No. 7, 32-34 (2013).
7. E. S. Petrova, *Morfologiya*, **136**, No. 6, 8-19 (2009).
8. E. S. Petrova and V. A. Otellin, *Ontogenez*, **35**, No. 2, 118-123 (2004).
9. E. S. Petrova and E. I. Chumasov, *Tsitologiya*, **35**, No. 1, 59-64 (1993).
10. K. N. Yarygin and V. N. Yarygin, *Zh. Nevrol. Psikhiatr*., **122**, No. 1, 4-13 (2012).
11. J. C. Baez, S. Gajavelli, C. K. Thomas, *et al*., *Exp. Neurol*., **189**, No. 2, 422-425 (2004).
12. G. D. Das, *Neural Grafting in the Mammalian CNS*, Amsterdam; New York (1985), pp. 101-123.
13. R. M. Grumbles, Y. Liu, C. M. Thomas, *et al*., *J. Neurotrauma*, **30**, No. 12, 1062-1069 (2013).
14. T. Murakami, Y. Fujimoto, Y. Yasunaga, *et al*., *Brain Res*., **974**, Nos. 1-2, 17-24 (2003).
15. P. E. Phelps, R. P. Barber, L. A. Brennan, *et al*., *J. Comp. Neurol*., **291**, No. 1, 9-26 (1990).
16. P. E. Phelps and J. E. Vaughn, *J. Comp. Neurol*., **355**, No. 1, 38-50 (1995).
17. S. Walsh and R. Midha, *Neurosurgery*, **65**, No. 4, Suppl., A80-A86 (2009).
18. G. Xiong, N. Ozaki, and Y. Sugiura, *Arch. Histol. Cytol.*, **72**, No. 2, 127-138 (2009).