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DEVELOPMENTAL BIOLOGY

Neurons with Different Neurotransmitters in Embryonic Neocortical Allografts in the Rat Sciatic Nerve

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Abstract—Different subsets of interneurons in the Wistar rat neocortex and in neocortical transplants developing in a damaged nerve were identified by the following immunohistochemical markers: glutamate decarboxylase (GAD 67) for GABAergic nerve cells, NO-synthase (NOS) for NO-ergic neurons, choline acetyltransferase (ChAT) for cholinergic cells, and tyrosine hydroxylase for catecholaminergic structures. Twenty-eight days after surgery, individual GAD 67-ir, NO-ir, ChAT-ir, and very rarely TH-ir cells were detected in the graft. It was shown that the number of GAD 67-ir neurons per unit area in the grafts was less than in the rat neocortex P20.

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INTRODUCTION

Currently, new cellular technologies intended to stimulate regeneration of damaged nerves are actively being developed in experimental studies (Walsh and Midha, 2009; Chelyshev, 2011; Fairbairn et al., 2015). According to some authors, the transplantation of neural stem/progenitor cells (NSPCs) (Murakami et al., 2003; Baes et al., 2004; Grumles et al., 2013), as well as fragments of embryonic anlages of the central nervous system (CNS) (Xiong et al., 2009), promotes the growth of nerve fibers in the recipient. It was shown earlier that rat anterior brain vesicle (ABV) fragments survive after transplantation into a damaged nerve, the transplanted progenitor cells differentiate into neurons and gliocytes, and the neuropil and a network of blood vessels are formed in transplants (Bernstein, 1983; Chumasov and Petrova, 1990; Petrova and Isaeva, 2014). The question of what types of nerve cells differentiate in embryonic neocortical grafts is little studied.

The aim of this work was to study the possibility of forming GABA(γ-aminobutyric acid)-ergic, catecholaminergic, NO(nitric oxide)-ergic, and cholinergic neurons in rat embryonic neocortical allografts developed after transplantation into a nerve.

MATERIALS AND METHODS

This study was performed with male ($n = 10$) and pregnant female ($n = 6$) Wistar rats. The animals were kept and euthanized in compliance with the rules of work with experimental animals (Annex to the Order of the Ministry of Health of the USSR no. 755 from December 8, 1977). Donors were represented by 14-day rat embryos, from which the fragments of the dorsolateral wall of the ABV containing neocortical anlages were isolated and transplanted under the perineurium of one of the sciatic nerve trunks of an adult rat. Dated embryos were obtained as described by Dyban et al. (1975). The sciatic nerves of recipient rats were preliminarily damaged by clamping for 40 s at the level of the upper third of the thigh. Near the site of damage, a small (1-mm) incision in the perineural and epineural membranes was made from the proximal segment of the nerve. The embryonic material was transplanted through an incision under the perineurium of the largest nerve trunk using a fine glass cannula (0.6 mm in diameter). The surgery was performed under a MBS-2 microscope (LOMO) (Chumasov and Petrova, 1990; Petrova and Isaeva, 2014). Twenty-eight days after the surgery, segments of the sciatic nerve with transplants were isolated and fixed in zinc–ethanol–formaldehyde (Korzhevskii et al., 2014). After appropriate treatment, the material was embedded in paraffin; immunohistochemical reactions were performed on paraffin sections 5 μm thick (Korzhevskii et al., 2014).

To study the nerve cells differentiating in transplants, we used mouse monoclonal antibodies to the neuronal nuclear antigen (NeuN) at a dilution of 1 : 400. The GABAergic cells were identified using rabbit polyclonal antibodies against the glutamate decarboxylase (GABA-synthesizing enzyme) isoform with a molecular weight of 67 kDa (GAD 67) (Spring Bioscience, United States) at a dilution of 1 : 100. The catecholaminergic neurons were identified using rabbit polyclonal antibodies to tyrosine hydroxylase (TH) (Abcam, United Kingdom) at a dilution of 1 : 500. The NO-ergic cells were identified using the antibodies to neuronal NO synthase (NOS) (Spring Bioscience) at

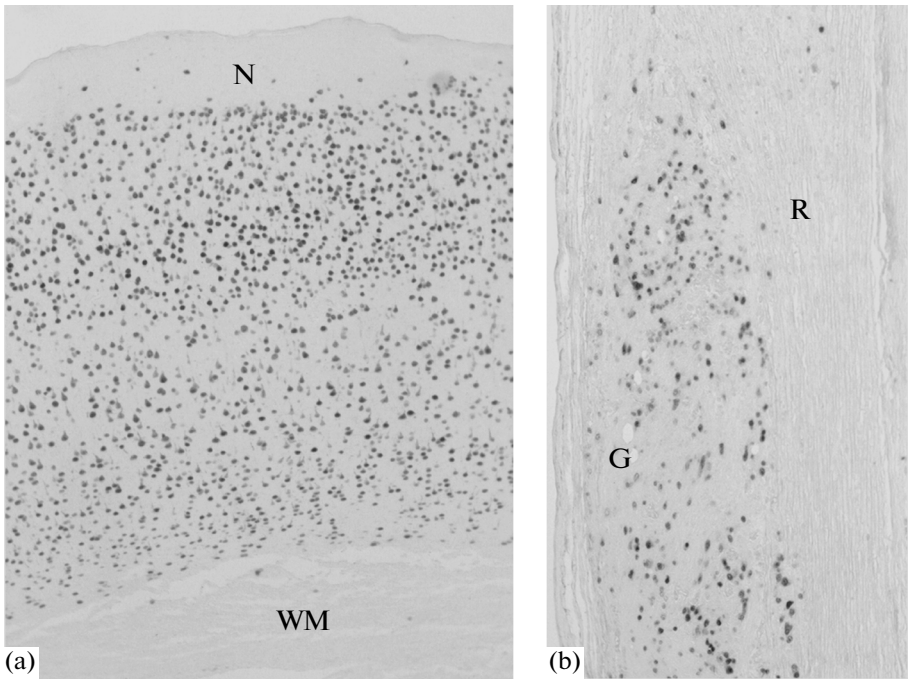

Fig. 1. Fragment of (a) rat neocortex (day P20) and (b) neurograft of a neocortical embryonic anlage 28 days after transplantation into the sciatic nerve of an adult rat. Designations: N—neocortex, WM—white matter, G—graft, R—recipient tissues. Expression of the NeuN marker. Magnification ×100.

a dilution of 1 : 1000. Reagents from the EnVision + System Labelled Polymer-HRP Anti-Mouse kit (catalogue no. K4001) (Dako, Denmark) and Super Sensitive Polymer-HRP Detection System (Bio Genex, United States) were used as secondary antibodies. Cholinergic neurons were identified with polyclonal goat antibodies to choline acetyltransferase (ChAT) (Chemicon, United States) at a dilution of 1 : 250. The antigen–antibody complexes were detected using secondary antigoat biotinylated antibodies (dilution 1 : 200, catalogue no. E0466) (Dako). Each reaction was performed simultaneously with an immunohistochemical negative control, in which the antibody diluent was applied on sections instead of the primary antibodies.

Some sections were stained with hematoxylin or toluidine blue. The morphological characteristics of the identified neurons of the grafts were compared to the interneurons of the somatosensory and motor cortex of rats at an age of 20 days (P20). Quantitative analysis of the cells expressing proteins NeuN, GAD 67, or NOS in the grafts and neocortex of rats on day P20 was performed on digital images obtained with a Leica DM 750 microscope (Germany) and a Leica ICC 50 digital camera (Germany). Cells were counted at a magnification of ×400 and ×1000 on five to ten images. Data were expressed per unit area of the graft. Calculations were performed using the ImageJ software (NIH, United States). Differences were estimated using Student's t test at $p < 0.05$.

RESULTS AND DISCUSSION

Twenty-eight days after surgery, tissue neurografts of embryonic anlages can be easily identified in the nerve. They consist of a large number of nerve and glial cells and neuropil and contain blood vessels. Using the NeuN nuclear antigen, an immunohistochemical marker, we showed that a large number of neurons is differentiated both in the grafts and in the rat neocortex developing in situ (Figs. 1, 2a). However, the number of cells expressing the markers of cholinergic, catecholaminergic, NO-ergic, and GABAergic neurons is small. To assess their differentiation, we performed a comparative study of neurons with the same neurotransmitters developing in the neocortex in situ.

It should be noted that the major part of nerve cells in the neocortex of vertebrates is represented by the excitatory glutamatergic neurons (Obukhov, 2008) and is not considered in this paper. To study their neurotransmitter activity after transplantation into the nerve, special research is required.

Interneurons account for 20–30% of the total number of neurons of the rat cerebral cortex and are very important for the normal functioning of the brain, as indicated by the development of neurological disorders as a result of disturbances in the interneuronal network (Reynolds et al., 2002; Levitt et al., 2004; Rajkowska et al., 2007). In the rat neocortex, interneurons form a heterogeneous population, the composition of which is determined by a complex of mor-

phological, cytochemical, and physiological characteristics (DeFelipe, 1993; Cauli et al., 1997; Markram et al., 2004; Obukhov, 2008; Asmus et al., 2011; Zaitsev, 2014). By the secreted neurotransmitters, they are divided into the GABAergic, cholinergic, catecholaminergic, and NO-ergic cells and neuropeptide-expressing cells. Some neurotransmitters colocalize in interneurons.

Immunohistochemical study of the motor and sensorimotor cortex of the rat brain on day P20 showed that the largest group of interneurons consists of the GABAergic cells, which are known to be inhibitory and localize not only in the neocortex but also in many structures of the brain (Cauli et al., 1997; Obukhov, 2008). It was shown that GAD 67 is present in the bodies, dendrites, and axonal terminals of GABAergic nerve cells (Wu et al., 1973; Petrova et al., 2013; Hozhai and Otellin, 2014). In the rat neocortex, GAD 67-immunopositive (GAD 67^+) cells were present in all layers. Some of them had processes (Fig. 3a). In neurografts, 28 days after surgery, a small number of GAD-67-containing neurons was differentiated (Fig. 2b). They were represented primarily by rounded cells with a narrow cytoplasmic rim (Fig. 3b). Occasionally, their processes could be revealed. The neuropil of grafts contained only single fragments of GAD 67-containing nerve fibers.

It is known that, in contrast to the excitatory glutamatergic neurons originating from the cells of the ventricular zone of the dorsolateral cell of the ABV, the GABAergic inhibitory interneurons of the neocortex are formed outside of this anlage, in the ventral region of the ABV (Obukhov, 2008; Bartolini et al., 2013; Le Magueresse and Monyer, 2013). The progenitors of these neurons perform a long-distance migration into the dorsolateral wall of the ABV for a long period of embryonic development, from E14 to E19 (Bartolini et al., 2013; Le Magueresse and Monyer, 2013). Since, to perform transplantation, we isolated a neocortical anlage on day E14, some progenitors of the GABAergic neurons, which migrate into the dorsolateral wall of the ABV later, did not get into the grafts. Thus, a possible cause of the small number of $GAD67^+$ neurons formed in the grafts is the peculiar origin of these cells in embryogenesis.

The rat cortical interneurons expressing NO-synthase on day P20 were identified primarily in the lower layers of the neocortex. Many of them had thick (up to 2 μm m thick in the proximal part) fairly long processes. In some of the NO-ergic cells, fragments of processes could be traced to distances over 50 μm (Fig. 4a). It was found that, 28 days after surgery, NOS-containing neurons were formed in the neurografts. Only some of these cells had processes, whereas the majority of them were represented by round or oval cells (Fig. 4c). The determination of the number of NOS^+ neurons per section unit area showed that the density of such neurons in the grafts was similar to their density in the rat neocortex on day P20 (Fig. 2c). Differentiation of NO-ergic neurons in the tissue grafts developing in the nerve was observed in our earlier study with the use of the histochemical reaction for NADPH diaphorase (Petrova and Otellin, 2000). The diversity of functions of the NOS-containing cells in the brain was described in several reviews (Reutov et al., 1998; Motavkin and Dyuizen, 2003; Obukhov et al., 2011). One of the functions of the gaseous neurotransmitter NO is the regulation of blood flow in the brain. The NO-ergic neurons differentiated under conditions of transplantation presumably can affect the blood supply of the grafts and, as a result, the survival of cellular elements in them.

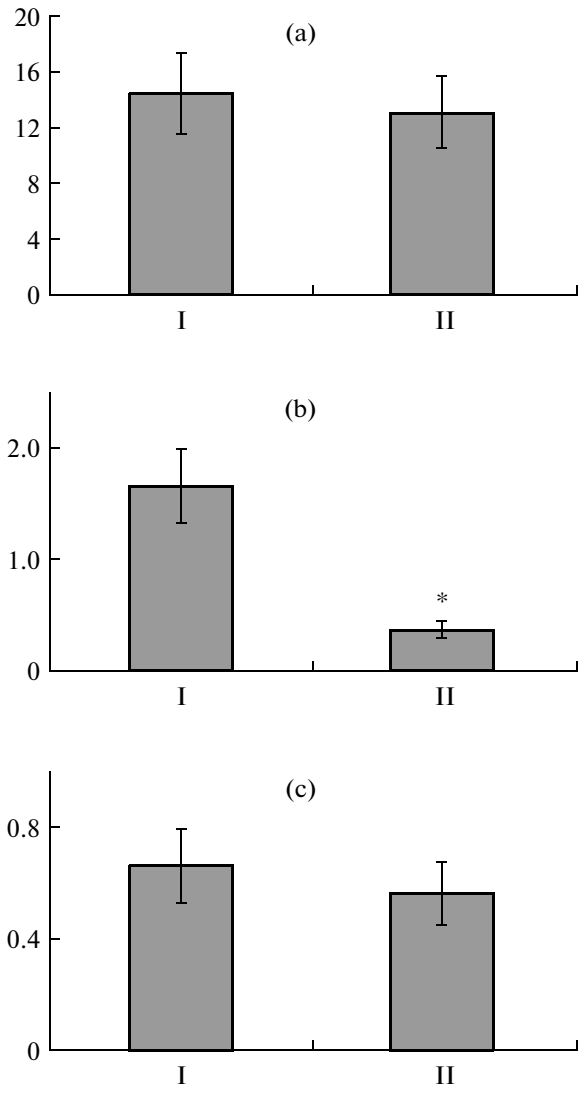

Fig. 2. Number of neurons containing (a) NeuN, (b) GAD 67, and (c) NOS proteins per section unit area in (I) the rat neocortex on day P20 and (II) in the neurograft. * $p < 0.05$.

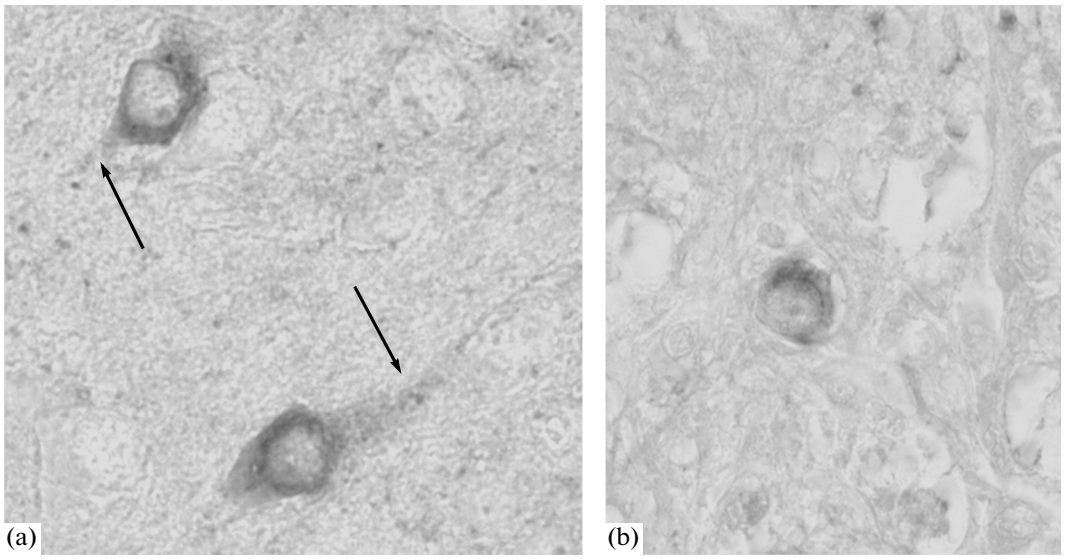

Fig. 3. GABAergic neurons in (a) the rat neocortex and (b) the graft of the neocortical embryonic anlage in the nerve. Arrows show the processes of neurons. Expression of the GAD 67 marker. Magnification ×1000 (for Figs. 3–5).

Although individual cholinergic neurons were detected in all layers of the rat cortex on day P20, the majority of them were concentrated in the upper layers. Here, they were usually bipolar and had long processes (70–80 μm) directed perpendicular to the brain surface (Fig. 5a). Despite the fact that the cholinergic neurons in the cerebral cortex of the mammalian brain were first described in 1980–1990 (Houser et al., 1985), their functions in the rat neocortex are not completely clear. It was assumed that they are modulating neurons and regulate the activity of other nerve cells and are involved in the regulation of blood flow and in the maturation of neocortical neurons in the ontogeny (von Engelhardt et al., 2007; Consonni et al., 2009).

In the neurografts of embryonic anlages, ChAT-containing cells occurred rarely. Their processes were usually not detected (Fig. 5b).

One of the most important afferent systems of the cerebral cortex is the catecholaminergic system. In all layers of the rat cerebral cortex, thin TH-immunopositive nerve fibers were found in cells located in the midbrain and medulla oblongata (Fallon, 1981; Grigor'ev et al., 2011). Individual TH-containing neurons were shown to be located in the upper and middle layers of the neocortex (Grigoriev et al., 2012). We also identified these cells in the neocortex of rats on day P20. In the neurografts, TH-immunopositive neurons were practically absent. In only one case was one TH-containing cell detected.

Twenty-eight days after surgery, a neuropil forming between the nerve and glial cells could be seen in neurografts. Using immunohistochemical markers, it was shown that the neuropil in the histological sections of the grafts contained the fragments of nerve fibers belonging to nerve cells with different neurotransmitters. Fragments of long processes of NO-ergic nerve cells could be traced to a distance of 50 μm. It should be noted that such fibers had small varicose swellings (Fig. 4d). Long NOS-immunopositive processes of a similar diameter with varicose swellings were also found in the neocortex of rats on day P20 (Fig. 4b). GAD 67-immunoreactive fibers were rarely detected in the neuropil. Among the ChAT-immunopositive fibers, processes 3–4 μm in diameter could be seen in the thickness of the grafts. These thick nerve fibers belonged to the recipient. Occasionally, thin filaments (not more than 1 μm in diameter) with rare varicose swellings were observed in the thickness of the grafts. These processes might belong to the single cholinergic cells that were identified in the grafts 28 days after surgery.

Catecholaminergic nerve fibers were detected in the grafts very rarely. Single TH-containing terminal endings could be seen near the blood vessels. Most likely, these nerve endings belonged to the recipient: it is known that, in addition to the cholinergic nerve fibers, peripheral conductors contain autonomic catecholaminergic axons. One of the function of these fibers is the vasa nervorum innervation (Nozdrachyov and Chumasov, 1990). Presumably, the catecholaminergic nerve fibers can get into the graft, accompanying the blood vessels of the nerve of the recipient growing into the graft.

One of the mechanisms of the beneficial effect of cell therapy on the regeneration of a damaged nerve is

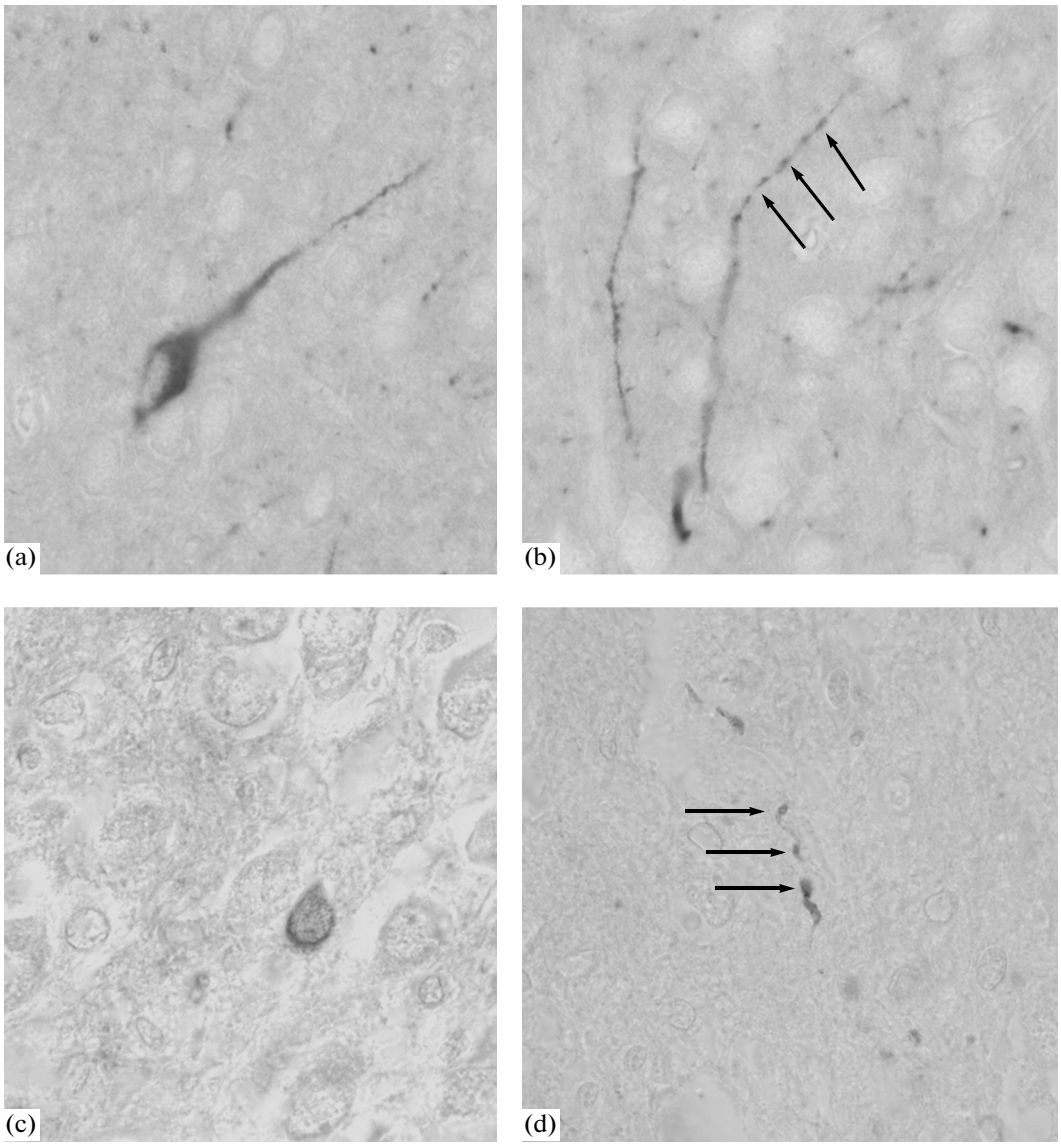

Fig. 4. Expression of the NO-synthase marker in (a, b) rat neocortical cells and (c, d) the graft of the neocortical embryonic anlage in the nerve. Arrows show the varicose swellings of nerve fibers.

the development and release of neurotrophic and growth factors by the transplanted cells (Murakami et al., 2003; Baez et al., 2004; Walsh and Midha, 2009; Fairbairn et al., 2015). The role of neurotransmitters of the transplanted cells is not taken into account in this case. However, as was shown in our work, the axons of some nerve cells, which were detected in the neuropil of the grafts, had varicose swellings. It is known that, in such swellings, neurotransmitters can be released from the cell immediately into the extracellular space without synapse formation (Vizi and Kiss, 2004; Zhang et al., 2011). In our opinion, they could be involved in the formation of a microenvironment for the regenerating fibers of the recipient and affect their growth.

CONCLUSIONS

Single GABAergic, NO-ergic, cholinergic, and catecholaminergic neurons were formed 28 days after surgery in the grafts of embryonic neocortical anlages developing in the nerve. Therefore, in a changed microenvironment after the transplantation into the nerve, some progenitor cells can differentiate into the neurons of the neurotransmitter profile characteristic of the neocortex. The comparative study of the neurons of neurografts and the interneurons formed in the rat neocortex in the respective period of ontogeny showed that the majority of cells of the grafts differed from the cortical neurons by the absence of processes. This might be due to the delay in their differentiation and the disturbance of formation of afferent and effer-

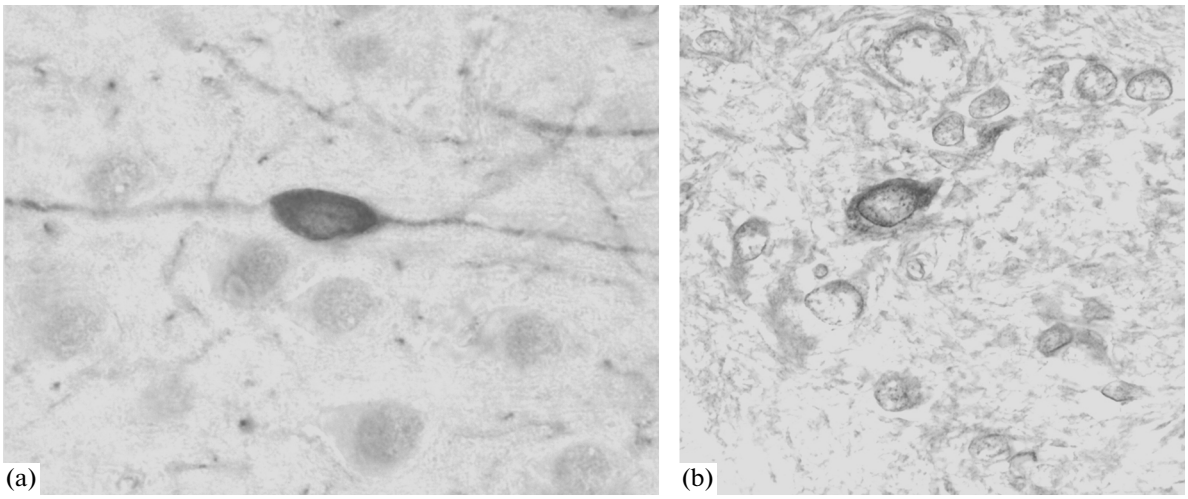

Fig. 5. Cholinergic neurons in (a) rat neocortex and (b) graft of the neocortical embryonic anlage in the nerve. Expression of the ChAT marker.

ent connections characteristic of the cortex. The density of the studied GABAergic neurons in the grafts was lower than in the rat neocortex on day P20, and $ChAT^+$ and TH^+ nerve cells were rare. Apparently, this was caused by disturbance of histogenetic processes in the grafts in the early period after transplantation.

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