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Differentiation and Cell–Cell Interactions of Neural Progenitor Cells Transplanted into Intact Adult Brain

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We studied the behavior and cell–cell interactions of embryonic brain cell from GFP-reporter mice after their transplantation into the intact adult brain. Fragments or cell suspensions of fetal neocortical cells at different stages of development were transplanted into the neocortex and striatum of adult recipients. Even in intact brain, the processes of transplanted neurons formed extensive networks in the striatum and neocortical layers I and V-VI. Processes of transplanted cells at different stages of development attained the rostral areas of the frontal cortex and some of them reached the internal capsule. However, the cells transplanted in suspension had lower process growth potency than cells from tissue fragments. Tyrosine hydroxylase fibers penetrated from the recipient brain into grafts at both early and late stages of development. Our experiments demonstrated the formation of extensive reciprocal networks between the transplanted fetal neural cells and recipient brain neurons even in intact brain.

Key Words: *neurotransplantation; undifferentiated cells; GFP mouse; cell processes; immunohistochemistry*

Neurodegenerative diseases and brain injuries are prevalent pathologies leading to serious and irreversible consequences. Limited regeneration potential of adult CNS can be determined by endogenous molecular properties of cell and inhibitory factors of the microenvironment [12,15], in particular, molecules produced by oligodendrocytes and blocking the growth of nerve cell processes (myelin-associated inhibitors) and extracellular matrix proteins of the chondroitin sulfate proteoglycan family (CSPGs). In case of injury, activated astrocytes and microglial cells also contribute to this inhibition. Astrocytes form a physical barrier, glial scar, containing CSPGs [12], while microglia activates immune response. However, the reaction of glial and immune response to trauma have also a positive consequences: monocytes exhibit anti-inflammatory activity, macrophages can synthesize neurotrophic factors stimulating repair, astrocytes and glial barrier protect neurons from NO and other toxic agents [4,19,20,22]. Nevertheless, pronounced restoration of the nervous tissue in adult mammals and humans does not occur.

At the same time, detection of neural stem cells unambiguously indicates that neurogenesis maintaining homeostasis and promoting local regeneration occurs in adult brain, at least in two zones. Moreover, it was found that injury stimulates proliferation of stem cells and newly formed cells can migrate to the damaged area. However, only a small portion of these cells can survive and differentiate into neurons [21]. Hence, endogenous neurogenesis cannot provide cell replenishment and functional recovery after injury.

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Transplantation of stem and progenitor cells for the treatment of neurodegenerative diseases is a promising trend in regenerative medicine. Progenitor cells can proliferate, adapt to novel conditions, and differentiate into various neural phenotypes, which opens great prospects for their medical and biological application. It is known that transplanted tissue can integrate into the recipient brain tissue and restore lost functions [13,18]. A wide range of neuron-like cells, the possibilities of their integration, and their effects on the recipient brain are now intensively studied. Neural cells derived from embryonic stem cells and induced pluripotent stem cells have great potentialities, but the risk tumor formation cannot be excluded [11]. In case of mesenchymal stem cells, complex and long-term culturing is required for induction of desired differentiation. In comparison of differentiation capacities and integration of cells obtained by using biotechnological approach only fetal brain cells can serve as the appropriate control, because they are native and possess no tumorigenic properties [11,16] and therefore are considered gold standard by many authors [16]. Despite ample knowledge about the development of fetal cells in the recipient brain, modern methods provided more detailed and comprehensive information on their differentiation and cell-cell communication.

Our aim was comparative immunohistochemical analysis of cell interaction in solid tissue and suspension allogeneic transplants of embryonic neural tissue from GFP-mice at different stages of development with intact brain tissue of the recipient.

MATERIALS AND METHODS

The experiment was carried out in accordance with the requirements of Bioethics Committee of V. I. Kol'tsov Institute of Developmental Biology. Adult 4-5-month-old female C57Bl/6 mice (n=35) were used as recipients. The animals were anesthetized with chloral hydrate (350 mg/kg) and placed in stereotaxis. Operation field was prepared and a hole was drilled +0.45 mm from bregma and 2 mm laterally.

The cells for transplantation were obtained from E12.5, E14.5, E19.5 embryos of transgenic GFP mice C57Bl/6-Tg (ACTB-EGFP)1Osb/J [17]. The embryonic neocortex was isolated in Hanks saline (Gibco). A fragment of neocortex was taken with a syringe with glass needle and stereotactically injected into the brain of the recipient through the hole to a depth of 2.5 mm, so that the graft spanned the cortex, striatum, and corpus callosum (Fig. 1, *a*). The operation wound was sutured. For transplantation of cell suspension, the fragment of the neocortex was dissociated by pipetting in accutase solution (Sigma-Aldrich) and then the cells were pelleted by centrifugation. The cells were counted in a Goryaev's chamber. The transplantation dose was 500,000 cells in 1.5 μl Hanks saline per mouse. The cells were transplanted with a Hamilton syringe as described above.

In 7, 30, and 60 days after transplantation, the animals were transcardially perfused with 4% paraformaldehyde in phosphate buffer (0.01 M PBS). The brain was removed and stored in a cryoprotector (30% sucrose) for 24 h. Sagittal brain sections (40 μ) were sliced on a freezing microtome. For immunohistochemical staining, brain sections were incubated for 24 h in 0.3% Triton X-100, 10% normal goat serum (Sigma-Aldrich), 0.01 M PBS (pH 7.4) with primary antibodies. For double staining, anti-GFP antibodies (1: 500, Molecular Probes) were used in combination with anti-GFAP (1:600, Abcam) for detection of astrocytes, anti-NeuN (1:100, Chemicon) for detection of differentiated neurons, anti-Ki67 (1:100, Abcam) for detection of proliferating cells, anti-DCX (1:300, Abcam) for detection of immature neurons, and anti-TH (1:100, Abcam) for detection of tyrosine hydroxylase fibers. After washing in PBS, the sections were incubated in 0.3% Triton X-100, 0.01 M PBS (pH 7.4) with secondary goat anti-rabbit or anti-mouse antibodies (1:500, Alexa fluor 568, Molecular Probes) and goat anti-chicken antibodies raised in goat (1:500, Alexa fluor 488, Molecular Probes). The analysis was performed using a Keyence BZ-9000E fluorescent microscope and Leica TCS SP5 laser scanning confocal microscope.

RESULTS

We studied the interaction of transplanted low-differentiated nervous tissue (neocortex obtained at different stages of embryonic development) with the brain tissue of an adult recipient. Transplants were either fragments (pieces) or a suspension of embryonic nervous tissue from reporter transgenic GFP mice, which allowed accurate visualization of donor cells and their processes in the recipient tissues. Histological analysis performed on days 7, 30 and 60 after surgery showed that the grafts were similarly located in the brain of the recipients spanning through the neocortex, corpus callosum, and striatum. Despite some grafts contained groups of dead cells and cyst, donor cells successfully survive in the recipient tissue throughout the observation period (60 days).

Analysis of differentiation of E12.5 embryonic cell (early neurogenesis). In 7 days after transplantation, the cells in tissue and suspension transplants differentiated towards glial lineage cells (Fig. 2, *a*), though differentiated neurons were absent. It is known that neuronal differentiation normally precedes glial differentiation, the latter peaks during the postnatal

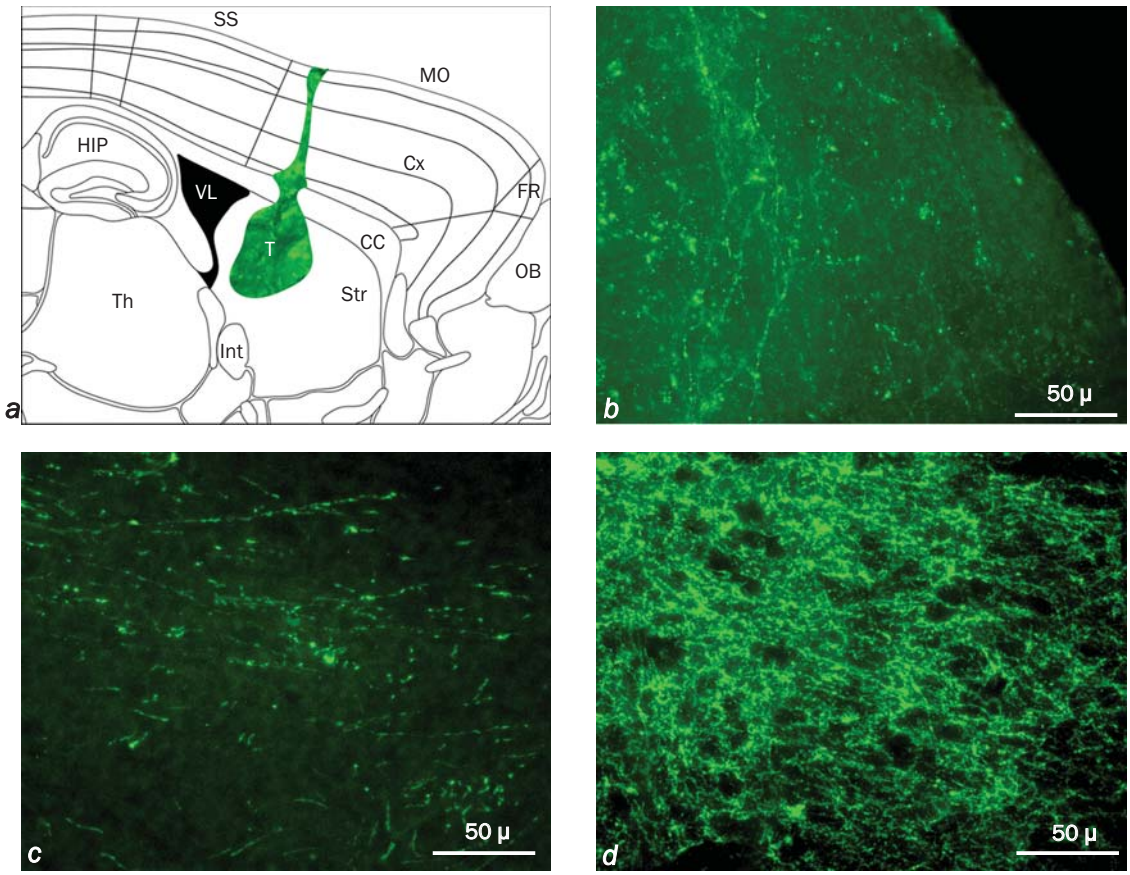

Fig. 1. Processes of transplanted cell in host brain structures. Immunohistochemical staining with antibodies to GFP. *a*) Schematic presentation of transplantation area. T: transplant, Cx: cortex, MC: motor cortex, SS: somatosensory cortex, Str: striatum, Int: internal capsule, Th: thalamus, HIP: hippocampus, OB: olfactory bulb, CC: the corpus callosum, FR: frontal pole, VL: lateral ventricle. *b*) Processes of transplanted in the frontal cortex of the host Suspension transplant of the neocortex from E12.5 embryo in 60 days after transplantation. *c*) Processes of transplanted cells in host internal capsule. Suspension transplant of the neocortex from E12.5 embryo in 7 days after transplantation. *d*) Processes of transplanted cells in host striatum. Tissue transplants of the neocortex from E12.5 embryo in 60 days after transplantation.

period. Hence, transplantation of the tissue from early embryos results in inhibition of neuronal differentiation and acceleration of glial differentiation. This can be related to microenvironmental influences from the adult brain and the transplantation procedure The cells in tissue and suspension transplants actively proliferate, which is typical of cells at the stage of early neurogenesis. Morphologically, the cells have irregular astrocyte-like or round shape. Among tissue transplants, cells resembling radial glia were noted. Migrating cells were primarily located along the fibrous tracts of the striatum and blood vessels of the recipient. After transplantation of the tissue fragments, we observed cells migrating under meninges and in the first layer of the cortex in the recipient brain. It should be noted that during normal brain development, cell proliferation and migration are typical of the initial period of neurogenesis.

Damage associated with transplantation and the foreign tissue stimulated glial reaction. Astrocytes of the recipient brain started expressing GFAP, but neither fragment not after suspension transplantation led to glial scar formation.

The use of antibodies to GFP enabled clear visualization of donor cell processes growing into the recipient brain tissue. After transplantation of tissue fragment, solitary cell processes were seen around the graft (within 300 μ). Fiber density was higher in the striatum, where they form dense bundles growing along the axonal tracts. It is noteworthy that on the sagittal sections we observed virtually no cell processes dorsoventrally crossing the corpus callosum; only rare fine fibers were found, though in our previous work we observed fibers spreading in the corpus callosum in the lateral direction and reaching the contralateral hemisphere on frontal sections [1].

A similar picture was observed after transplantation of cell suspension. Donor cell processes extended within 500 μ and formed bundles accompanying the striatum fibers in the recipient brain. Some projec-

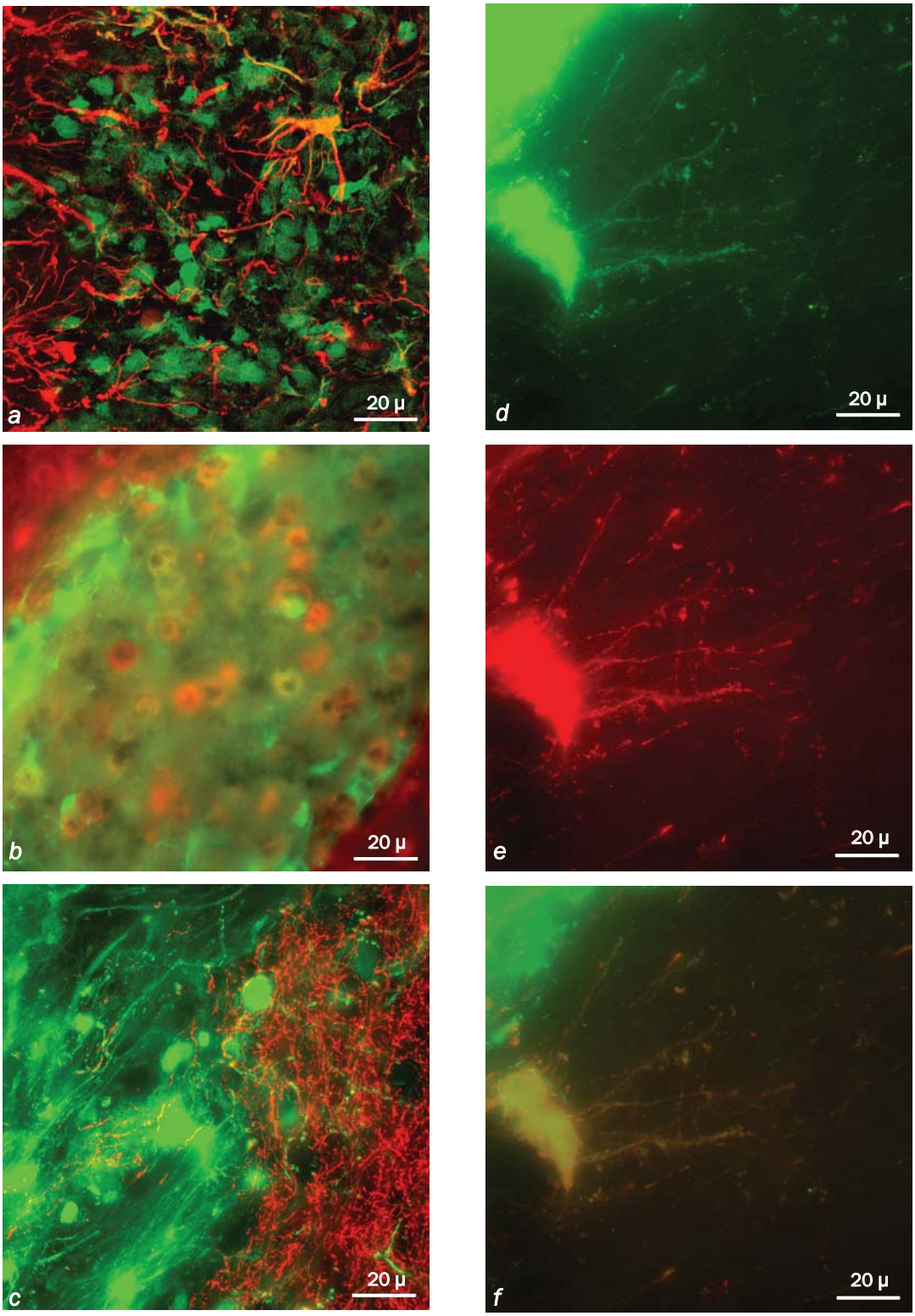

Fig. 2. Differentiation and reciprocal growth of transplant and host cell processes. *a*) Glial differentiation of transplanted cells. Suspension transplant of the neocortex from E12.5 embryo in 7 days after transplantation. Staining with antibodies to GFP and GFAP. *b*) Neuronal differentiation of transplanted cells. Tissue transplants of the neocortex from E14.5 embryo in 30 days after transplantation. Staining with antibodies to GFP and NeuN. *c*) Penetration of host TH fibers into the transplant. Tissue transplants of the neocortex from E12.5 embryo in 60 days after transplantation. Staining with antibodies to GFP and TH. *d-f*) Neural cell processes in the transplant. Tissue transplants of the neocortex from E12.5 embryo in 7 days after transplantation. Staining with antibodies to GFP and DCX.

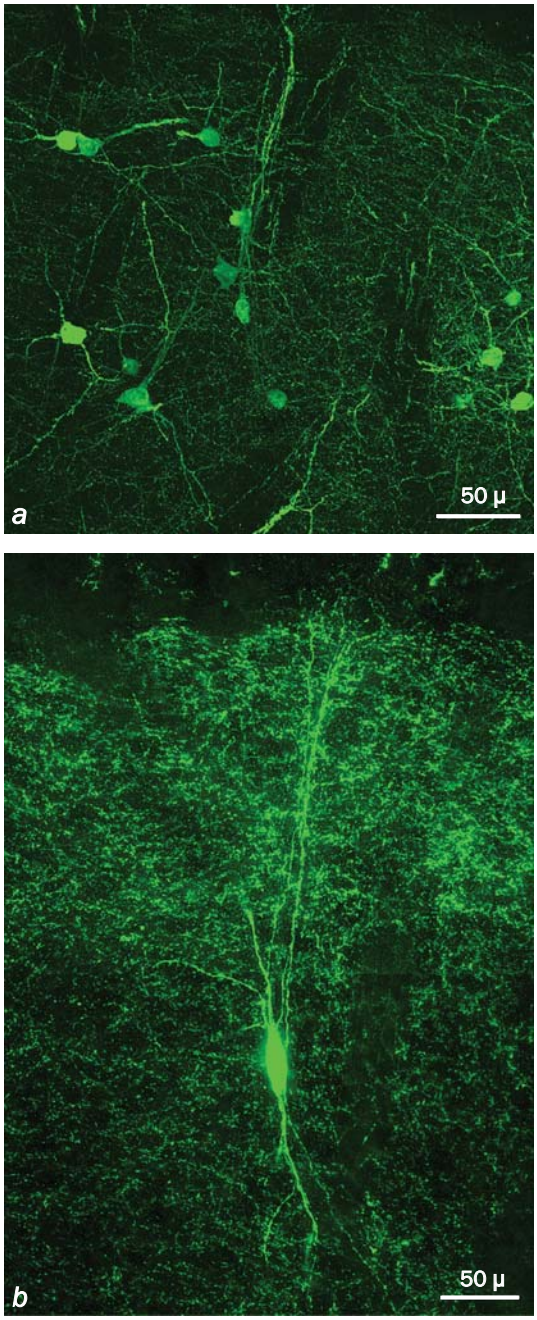

Fig. 3. Morphology of cells migrating from transplant and located among dense fiber network in the host striatum. Immunohistochemical staining with antibodies to GFP. *a*) Cells migrated from tissue transplant of the neocortex of E12.5 embryo in 30 days after transplantation; *b*) a cell migrated from the suspension transplant of the neocortex of E14.5 embryo in 60 days after transplantation.

tions reached internal capsule (Fig. 1, *c*), which can indicate the formation of connections with subcortical structures. Cell processes from the transplants (solid tissue and suspension) primarily grew in the rostral direction. No synaptic spines were found on dendrites.

According to immunohistochemical analysis data, in 30 days after transplantation, the cells in solid tissue transplants differentiated into neurons, whereas cells in suspension transplants were characterized by considerably suppressed NeuN expression (a marker of mature neurons). This can be a result of cell damage during suspension preparation, because expression of NeuN decreases after injury to the brain tissue and neural cells cannot be identified by immunohistochemical methods [2]. In contrast to neurons, astrocytic differentiation was detected in both solid tissue and suspension transplants. Cell proliferation in grafts was practically absent. However, proliferation of endogenous stem cells in the subventricular zone and rostral migratory tract in the recipient brain was activated after fragment transplantation.

During this period, donor cells acquired morphology of pyramidal and stellate neurons and astrocytes (Fig. 3, *a*). Active migration of donor cells into the neocortex and striatum of the recipient was observed only in case of fragment transplantation. Glial reaction to the transplant in the recipient brain developed, but without the formation of a pronounced scar around the transplanted cells.

Cell processes from the solid tissue transplant formed a very dense network in the recipient brain tissue. Their density was maximum in the upper region of the striatum (under the corpus callosum) and in layers I and V-VI of neocortex. Processes, primarily radially oriented fibers, extend to rostral (1.7 mm) and caudal (1.1 mm) directions from the transplant, but did not cross the corpus callosum dorsoventrally. After transplantation of the cell suspension, the processes did not form dense network. Dense bundles along the striatum fibers and individual processes in the VI layer of the recipient neocortex were revealed. Dendrites of cells of solid tissue transplants carried spines, which indicated the formation of synaptic contacts with neurons in the recipient brain.

In 60 days after transplantation, the cells in solid tissue and suspension transplants differentiated into neurons and astrocytes. No proliferating cells were detected. Cells had morphology of neurons and astrocytes. Only solitary migrating cells were seen. The transplants were not rejected, no glial scar was formed. The pattern of cell process distribution in suspension and solid tissue transplants on day 60 after surgery was similar to that in solid tissue grafts on day 30. The highest density of processes was found in the upper regions of the striatum (Fig. 1, *d*). Interestingly, cell processes in the suspension transplant extended rostrally to deep layers of the frontal cortex (Fig. 1, *b*). Thus, differentiation of cells of suspension transplants from E12.5 embryos was delayed and the network of processes similar to that formed by tissue transplants appeared only on day 60 after surgery.

Differentiation of embryonic E14.5 cells. In 7 days after surgery, differentiated neurons expressing

NeuN were absent in both types of grafts, though differentiated donor glial cells were identified similar to transplantation of neocortical tissue from E12.5 embryo. Cell proliferation was lower than in E12.5 tissue transplants. Cells transplants usually have irregular shape. Active migration was observed near the transplant. After transplantation of solid tissue fragments, a dense bundle of migrating cells was formed in the upper part of the striatum (under the corpus callosum). Transplanted tissue caused glial reaction of the recipient, but no glial scar appeared.

Antibodies to GFP showed processes of donor cells in the recipient brain. In the case of solid tissue transplants, a dense network of processes in the striatum was formed as soon as on day 7 in culture, the density of processes was maximum in its upper areas. Some projections reached the neocortex layers I and V-VI in the rostral (by 1 mm) and caudal (500 mm) directions. Interestingly, single projections were observed in the internal capsule. In suspension transplants, only few processes spread along the fiber tracts of the striatum and internal capsule.

In 30 days after transplantation, the cells in solid tissue and suspension transplants differentiated into neuronal and glial cells (Fig. 2, *b*). The cells stopped proliferate and acquired typical morphology resempling pyramid and stellate neurons and glial cells. Solitary migrating cells were seen. Dendrites carried spines. Glial reaction was mild and glial scar was not formed. Dense bundles of cell processes grew from the solid tissue transplant along the fibers of the striatum in the recipient brain. Thin processes originating from cells of the suspension transplant primarily grew in the recipient brain along the fibers of the striatum.

In 60 days after transplantation, cells differentiated in neuronal and glial cells were found. Proliferation was absent similar to 30 days after transplantation. Solitary migrating cells were seen (Fig. 3, *b*). The tissue transplant was found in the ventricle, cell processes formed a network in the fornix of the recipient. Processes of cells in the suspension graft formed a dense network in the striatum and layer I of the cortex in the recipient brain, maximum density was observed in the upper region of the striatum under the corpus callosum. Solitary processes propagated from the transplant in layer VI of the neocortes by 600 μ in the caudal direction and reached the deep layers of the frontal cortex in the rostral direction.

Analysis of differentiation of E19.5 embryonic cell (differentiation of glia, neurogenesis is completed). In 7 days after transplantation, cells differentiated into neurons were identified in tissue transplants, while in cells of the suspension transplant, NeuN expression was suppressed. Donor cells also differentiated into glial cells. Only solitary proliferating cells were found. Some migrated cells were seen near the transplant. Cells of the suspension transplant migrated along the striatum fibers. No glial scar was formed around the transplant.

Despite the transplanted tissue was taken at the late stages of neurogenesis, extensive growth of processes was observed. In case of solid tissue transplant, the processes formed characteristic dense network in the upper region of the striatum. However, in suspension transplants, no fibers were observed.

In 30 days after transplantation, the neurons expressing NeuN and astrocytes were detected in both solid tissue and suspension transplants; proliferative activity was absent. Many cells had regular shape and resembled pyramid cells, though their structure was poorly discernible in the dense part of the tissue transplant. Cells of the suspension transplant were scattered. Cell migration from the transplant was extremely weak, which is natural for cells of the neocortex at the late developmental stage. Interestingly, pronounced glial scar was formed around the solid tissue transplant, but was absent around the suspension transplant.

The cell processes in the tissue transplant formed characteristic network with maximum density in the upper region of the striatum. The processes grew in layers I, and V-VI of the neocortex in the recipient brain and reached the frontal cortex in the rostral direction. Suspension cells formed only solitary processes in the striatum within 100 μ.

In 60 days after transplantation, the cells in both the solid tissue and suspension transplants were differentiated into neurons and astrocytes. Proliferation was absent. Many cells looked like differentiated neurons. Interestingly, transplantation of the tissue fragment was followed by extensive cell migration in the neocortex of the recipient, whereas only solitary cells migrated from the suspension transplant. It should be noted that the tissue transplant was surrounded by a dense glial scar, but it did not prevent the growth of processes. It can be hypothesized that glial barrier in this case was formed after the appearance of cell processes. The density of processes was high near the transplant and decreased with increasing the distance from it. The processes form a dense network in the upper regions of the striatum and layer I of the neocortex and reached the deep layers of the frontal cortex in the rostral direction. The pattern of process distribution in case of cell suspension was similar to that in the tissue transplant, although their growth was limited by 600-μ zone.

The observed network of processes originating from transplanted cells raised the question about their nature. To answer this question, we used antibodies to markers of neuronal and glial cells and GFP. GFAP (marker of astrocytes) was not expressed in cell pro-

cesses, hence, the fibers were not glial processes. Staining for DCX (doublecortin, marker of immature neurons) and GFP (cells of the transplant) showed that long processes contained both markers, which attested to their neuronal nature (Fig. 2, *d-f*). Interestingly, DCX expression in donor cells detected in 7 days after transplantation was typical of both transplants irrespective of the age of embryonic nervous tissue. Since DCX is expressed in immature low-differentiated neuronal cells capable of migration, it is not surprising that its expression disappeared in 30 days after maturation of neurons in the transplants.

Along with abundant growth of fibers from transplanted cells into the recipient brain tissue, processes of tyrosine hydroxylase (TH) cells of the recipient brain can penetrate into the transplanted embryonic neocortical tissue and form contacts on donor cells (Fig. 2, *c*). TH fibers of the recipient grew into both tissue and cell transplants of different developmental stages, and their density correlated with that in the recipient brain. Since the density of TH fibers is low in the neocortex and high in the striatum, the part of the transplant located in recipient's striatum was more saturated with TH fibers than the part located in the neocortex.

The interaction of transplanted low-differentiated cells with the recipient tissue is a very important question. Modern technologies provide tools for visualization of transplanted cells and their processes and allow evaluating the potencies of transplanted cells. The use of transgenic GFP mice allowed accurate detection of donor cells and their processes. Experiments with transgenic GFP mice unambiguously showed that donor GFP-labeled cells and recipient cells did not fuse [7,9].

It is obvious that cell transplantation into adult brain was followed by their adaptation to new environment accompanied by their significant loss over the first few days [23]. Moreover, cell survival can depend on the type of the transplant. For instance, experiments with transplantation tissue fragments or cell suspension from human fetal midbrain into 6-hydroxydopamine-lesioned rat brain [5] showed considerably broader age limit for transplantation of solid implants in comparison with suspension grafts. Our results agree with these findings. Although we traced the transplanted cells for only 60 days, it is known from experimental studies and even clinical observations that fetal grafts can survive for more than 18 years [10].

It is commonly accepted that cells isolated from early embryos more successfully integrate after neurotransplantation. Our experiments showed that brain tissue from fetuses at later developmental stages when neuronogenesis is completed can also be used for transplantation. This was also noted by other authors. For instance, comparative analysis [6] of the interaction of the suspension transplants of the neocortex from E17 and E19 mouse embryos with recipient brain undergoing apoptotic neuronal degeneration of callosal projection neurons in neocortex layers II, III, and V showed migration of transplanted cell to neocortex layers II, III, and V and NeuN expression (marker of differentiated neurons) in 66% of these cells in 6 weeks after transplantation. In the control, the same cells transplanted into intact brain demonstrated weaker migration and yielded only 10% differentiated neurons. As we studied neuronal differentiation of transplanted cells within shorter time intervals, neurons expressing NeuN were found on day 7 after transplantation only in neocortical tissue transplants from E19.5 embryos. In suspension transplants of the same age, no NeuN expression was found, which was probably a result of damage during preparation of the suspension [2,23].

In almost all cases, accelerated glial (astrocytic) differentiation of transplanted neocortical cells was observed. This was most pronounced in transplants from early embryos. Thus, E12.5 neocortex tissue in 7 days after transplantation attained the age of 19.5 days and demonstrated highly pronounced glial differentiation. In the intact neocortex of the same age, $GFAP^+$ glial cells were not detected and the peak of gliogenesis generally falls on the postnatal period.

Our study has demonstrated that donor cells form long processes that extensively grew into the intact (not previously injured) brain tissue of the recipient. These findings contradict the results of previous studies showing that cell processes after transplantation into the intact brain demonstrate only minimum growth [6] and can reach only the adjacent parts of the recipient cortex, but not distant targets [8]. It is noteworthy that the distribution pattern of cell processes in the transplants in the intact brain observed by us was similar to that observed after cell transplantation into damaged brain. For instance, after transplantation of motor cortex fragments from E14 mouse embryo into a lesion cavity in the cortex [9], the maximum density of processes from transplanted cells were found in the dorsal part of the striatum, layers V-VI of the neocortex, and internal capsule of the host. Some projections were found in the thalamus, contralateral cortex, and spinal cord. This extensive growth can be explained by induction of various signal molecules and demyelination accompanying traumatic lesion and contributing to the formation of regeneration-stimulating microenvironment [6,8].

We found that cells in suspension transplants form extensive fiber network later than solid transplants. Moreover, cells in suspension transplants from embryos at later development stages formed shorter processes. However, some authors observed that cell transplants from embryos at late developmental stages formed greater number of long processes than cells obtained at early stages of embryo development [6].

According to our data, processes of the transplant had neuronal nature, because expression of DCX, the marker of early migrating neurons, was observed as soon as on day 7 after transplantation in both cells and processes fibers. In 30 days, expression of this marker disappeared and the cells started expressing NeuN, marker of mature neurons. A similar differentiation trend was described after transplantation into damaged neocortex [9].

In parallel with the growth of cell processes from the transplant, host cells also form processes integrating into the donor tissue. We found that the neocortical transplant tissue does not block, but even stimulates ingrowth of host TH fibers. Moreover, TH fibers form synapse-like structures on transplanted cells. We also observed spines on the dendrites of transplanted cells, suggesting possible synaptic contacts. This is consistent with the results of previous studies demonstrating the possibility of synapse formation and electrophysiological activity of cells [9]. Hence, low-differentiated transplant provides a permissive microenvironment for reciprocal growth of processes, which confirms the possibility of mutual integration of the donor and recipient [3].

The study of glial reaction to transplantation in the recipient brain revealed similar intensity of gliosis in all experiments, while glial scar was formed only on day 60 after transplantation of embryonic neural tissue at the late development stage. It is important to note that the scar did not prevent the formation of a dense network of processes from the transplant. Other authors also reported that the formation of glial scar surrounding the transplant did not prevent the growth of processes in transplanted cells [7]. This can be explained by more rapid growth of processes forestalling glial scar formation, due to which glial scar components can act not only as inhibitors, but also as stimulators of regeneration [14].

Thus, our experiments showed that intact adult brain despite inhibitory microenvironment possesses high potentials for integrative interaction with transplanted undifferentiated cells of fetal neocortex. Reciprocal growth of cell processes occurs between the transplant and host tissue. Transplanted neurons extend numerous processes and the pattern of their growth is similar to that observed in brain injury models [6,8,9]. The most preferred targets are dorsal striatum and layers I and V-VI of the neocortex. We also showed that the brain tissue at late stages of embryonic development is suitable for transplantation and differentiated neurons present in the transplant can integrate with the host brain tissue. Solid transplants more rapidly adapted and integrated than suspension transplants. This can be explained by preserved microenvironment and cell-cell connections in the solid transplants. The observed integrative interaction between intact adult brain and donor fetal nervous tissue suggests that regenerative potential of mature CNS is underestimated.

Analysis of integration of native undifferentiated cells not only extends our knowledge about the regeneration capacities of CNS, but also helps to compare adequately different cell types intended for transplantation

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