Exp Brain Res 40, 265–272 (1980)

Experimental
Brain Research

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Transplantation of Embryonic Occipital Cortex to the Brain of Newborn Rats

An Autoradiographic Study of Transplant Histogenesis

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Summary. The neurogenesis of immature cerebral cortex transplants was investigated using tritiated thymidine (^{3}HT) autoradiography. Cortical tissue taken from rat fetuses during their last week of gestation (E15–E21) was transplanted to the tectum or cerebral cortex of newborn rat hosts. At different times after transplantation, a single injection of ^{3}HT was given to the host. Most of the experimental animals were killed after the transplants had grown to maturity (5–12 weeks), and some were studied shortly after the tracer had been given. In other experiments, donor tissue was used that was labeled in utero up to 1 day before being transplanted on E16, E17, E18, or E19.

It was found that neurons labeled before transplantation survived and differentiated in the graft. Removal of the graft from its natural context did not prevent ^{3}HT incorporation into surviving precursor neurons, indicating continuation of neurogenesis in the cortical transplants. In transplants from E16 donors neurons continued to be generated for 5–6 days after transplantation. Termination of neurogenesis occurred at successively earlier times in transplants taken from correspondingly older embryos. Independent of size and position of the transplant, application of ^{3}HT after "projected" transplant ages of E23 and older labeled only non-neuronal cells. This suggests a time schedule of neuron generation in the cortical transplants similar to that observed during normal development of the cerebral cortex, which is not disturbed by the transplanting procedure.

Key words: Transplant – Cerebral cortex (rat) – Neurogenesis – Differentiation – Autoradiography

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Formation of the cerebral cortex from the lining of the telencephalic vesicles proceeds in a very precise manner. Most neurons of the cerebral cortex are generated over a limited period of embryogenesis. Neurons undergoing their final mitosis at a given time during that generation period are destined to occupy a particular layer of the adult neocortex. This has been elegantly demonstrated using tritiated thymidine autoradiography in several mammalian species (*mouse* – Angevine and Sidman 1961; *rat* – Berry and Rogers 1965; Hicks and D'Amato 1968; for review see Berry 1974; *golden hamster* – Shimada and Langman 1970; *rabbit* – Fernandez and Bravo 1974; *monkey* – Rakic 1977). These studies have shown that except for layer I, which is one of the first formed cortical layers (Marin-Padilla 1971; König et al. 1977; Rickmann et al. 1977; Raedler and Raedler 1978; Goffinet and Lyon 1979), neurons in layers VI and V are born before those in layer IV and so on. This pattern is known as the inside-out formation of the cerebral cortex. Because precursor neurons undergo mitoses only in the region next to the ventricular lumen of the neural tube (His 1890; Sauer 1935), the postmitotic cells in the developing cerebral cortex, once generated, have to migrate to their respective position in a specific lamina (Berry and Rogers 1965; Rakic 1974; Seymour and Berry 1975; Shoukimas and Hinds 1978). X-ray radiation which eliminates dividing cells (Hicks et al. 1959, 1961; Altman and Anderson 1969) interferes with this process, leading to severe disruption of lamina and abnormal cerebral cortex development. In the reeler mutant mouse a disturbance of cell migration patterns during development of the cerebral cortex results in abnormal cell positioning (Caviness and Sidman 1973; Caviness and Rakic 1978).

During the course of studies in which we examined the formation of connections between cortical transplants and the host brain (Jaeger 1979;

0014–4819/80/0040/0265/$ 1.60

Jaeger and Lund 1978, 1979a, b, 1980), it became important to know whether the histogenesis of the cortical transplant was affected by removal from its natural context. Previous studies have shown that cerebral cortex from mammalian fetuses may survive transplantation (Dunn 1917; LeGros Clark 1940; Glees 1955; Das and Altman 1971; Das 1974, 1975) and that identified neurons of different types differentiate within these transplants (Das 1975, 1978; Jaeger and Lund 1978).

In the present study we address three main questions. First, do neurons that were generated prior to transplantation survive and differentiate in a cortical graft? Second, will transplanted precursor neurons give rise to neurons even though geometrical relationships were disturbed during the process of transplantation? Third, if neurogenesis continues, what is its time course in the cortical transplant? Some of this work has been reported previously in the form of an abstract (Jaeger and Lund 1978).

Materials and Methods

Occipital cortex taken from rat embryos (E) at gestational days E15–E21 was transplanted to either the cortex or the tectal region of newborn rats. The transplantation procedure was similar to that used previously (Lund and Hauschka 1976; Jaeger and Lund 1980). Time-mated albino rats of the Sprague-Dawley strain (Tyler Labs., Bellevue, WA, USA) were delivered of fetal donors under ether anesthesia by cesarean operation. The dorsal portion of the occipital cortex was dissected from the donor in tissue culture medium (F-10, GIBCO) and injected into the respective region of anesthetized newborn host rats.

A total of 72 experimental animals from 13 litters was injected once with 5 μCi/g b. w. tritiated thymidine (^{3}HT, specific activity, 50–65 Ci/mmol, New England Nuc.) on different days after transplantation. Fifty-two animals with noticeable transplants were used for this study. Table 1 shows a summary of the thymidine injection schedule and the number of animals processed in each of the seven different transplant groups. Four host animals in group II and six animals in group V were killed between 4 and 24 h after the ^{3}HT injection. Their brains were fixed in Bouin's fluid and 12 μm paraffin sections were cut. All other experimental animals were allowed to survive between 5 and 12 weeks after the procedure. They were perfused transcardially under deep anesthesia with a phosphate buffered (0.17 M) 4% paraformaldehyde solution. The position of the transplant was registered on a camera lucida drawing prepared under the dissecting microscope from the dorsal brain surface. Subsequently, the tissue was blocked, postfixed in the perfusion solution for at least 2 days, infiltrated with 30% sucrose, and cut on a freezing microtome at a thickness of 26 μm. Some material was embedded in egg yolk prior to cutting. For autoradiography (Sidman 1970) a series of sections (one in three, or one in four) was mounted on gelatin coated slides, coated with Kodak NTB-2 emulsion and exposed in the dark for an average of 4 weeks at 0° C before developing in Kodak D-19 and counterstaining with cresyl violet. In addition, a series of sections from each animal was stained for neurofibrils (Lund and Westrum 1966) to aid in identification of major fiber bundles and transplant borders.

Table 1. Shows numbers of animals with transplants that were analyzed in each group

Group	Age of Donor	Projected graft age at the time of ^{3}HT injection into the host										Total No.
		E15	16	17	18	19	20	21	22	23/0	1	
I	E15			2								2
II[a]	16			5	4	4	3	2	2	2		22
III	17					2	2	2	1		2	9
IV	18				2			2				4
V[a]	19						5	3	2		1	11
VI	20								2			2
VII	21										2	2

[a] groups in which some animals were killed after short survival

Additional material for this study was obtained from 50 further donor rats that received a transplant which had been prelabeled with ^{3}HT up to 1 day before transplantation on E16, E17, E18, or E19. These animals, some of which were also used for pathway studies reported elsewhere (Jaeger and Lund 1980), were processed as described above.

Results

Cortical transplants were identified overlying or embedded in the dorsal surface of the midbrain or alternatively embedded in the host cerebral cortex. Superficially placed myelinated fibers in the transplant aided in the recognition of the dorsal transplant boundaries in the surrounding gray matter of the host brain (Fig. 1A). In addition to the neurofibrillar staining, cytoarchitectonic criteria were also used.

Variability of transplant survival ranging between 30% and 100% was found to be highly correlative with two main factors. The first was related to technical proficiency gained over the course of this study, and the second to the age of the donor tissue. Most consistent graft survival (80% to 100% per litter) was observed for cortical transplants taken from either E15, E16, or E17 donors. In transplants taken from older embryos, particularly E20 and E21, survival to maturity was reduced to approximately 50% per litter.

A large number of host rats received transplants that were labeled in utero with ^{3}HT prior to transplantation on E15–E18. Autoradiograms from prelabeled cortical transplants that survived for periods of 1 week to 11 months contained neurons and non-neurons labeled with various intensities (Fig. 1B). Neurons were identified by their characteristic pale staining nuclei and their comparatively larger soma size. Similarly, some non-neuronal cells were identified based on their staining properties and morphological appearance (Hommes and Leblond 1967;

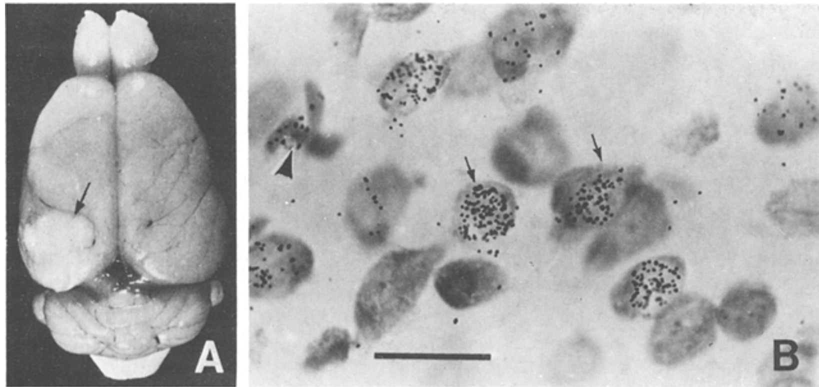

Fig. 1A. Dorsal surface of host brain showing cortical transplant (arrow) positioned in the occipital cortex. × 2. **B** Labeled neurons (arrows) and non-neurons (arrowhead) are observed in mature transplant subjected to ^{3}HT before transplantation (tracer applied on E16, donor tissue transplanted on E17). Note unlabeled neurons adjacent to labeled ones. Scale 25 μm

Table 2. Occurrence of labeled neurons in mature transplants subjected to ^{3}HT at different times after transplantation (post TR) – (+) occasional neurons labeled in some transplants

Day of ^{3}HT administration post TR	15	16	17	18	19	20	21
7		no	no				
6		no(+)					
5		+	no(+)		no		
4		+	+	no			
3		+	+		no(+)		no
2	+	+	+		+	no	
1		+			+		
				+			

Age of donor tissue at time of transplantation

Ling et al. 1973). No difference in labeling pattern was seen between transplants positioned in either the host cortex or tectum. Labeled neurons were found only within the confines of the transplant where they were scattered among unlabeled neurons (Fig. 1B) or localized in irregular clusters. In some cases few labeled glia cells were found in the surrounding host tissue but only in the immediate vicinity of the transplant. In an exceedingly small number of these transplants (< 1%) a considerable number of mitotic figures was observed, but no labeled neurons were seen. This finding was interpreted as a teratogenic transformation of the transplanted precursor cells in which the ^{3}HT tracer became diluted because of continued mitoses.

A summary of the cell labeling observed in the cortical transplants subjected to ^{3}HT at various times after transplantation is given in Table 2. Transplants that were taken from E16 donors and subjected to a single injection of ^{3}HT given from day 1 up to day 5 after transplantation showed labeled neurons. Neurons were marked with variable numbers of exposed silver grains. Their distribution in the transplant was patchy and more neurons were labeled in the grafts injected with ^{3}HT shortly after transplantation (e.g., up to day 3 post-transplantation). The same distribution was also noted in cortical transplants taken from E17 donors. As shown in Table 2, neuronal labeling in grafts from E17 donors was seen infrequently on day 5 post-transplantation and ceased completely after this time. In the younger transplants (E16) neurogenesis as observed by the uptake of thymidine stopped between days 6 and 7 after transplantation.

Figure 2 illustrates an example of the distribution of labeled cells in a mature cortical transplant taken from an E17 donor, placed into the tectum, and subjected to ^{3}HT 3 days after transplantation. Exposed silver grains overlying radioactive nuclei appear bright in darkfield illumination. The pia membrane covering the transplant surface is marked by an arrow, and the approximate position of the transplant/host border is indicated by circles. Regions containing many labeled cells (*) form a partially continuous band and patches in central and superficial parts of this transplant. The clusters contain labeled neurons and non-neurons which show variable amounts of silver grains suggesting different numbers of divisions subsequent to ^{3}HT administration (Figs. 4, 9). In areas containing few labeled cells, fiber bundles predominate over clusters of unlabeled neurons. Presumably unlabeled cells either failed to incorporate the ^{3}HT or underwent multiple divisions. Although groups of labeled neurons sometimes form sheets (Fig. 2), this tends to be rare. Labeled cells in the host brain are exclusively non-neuronal.

Mature transplants taken from E18 and E19 donors and labeled from days 0 (day of transplantation) to 2 days after transplantation showed a signific-

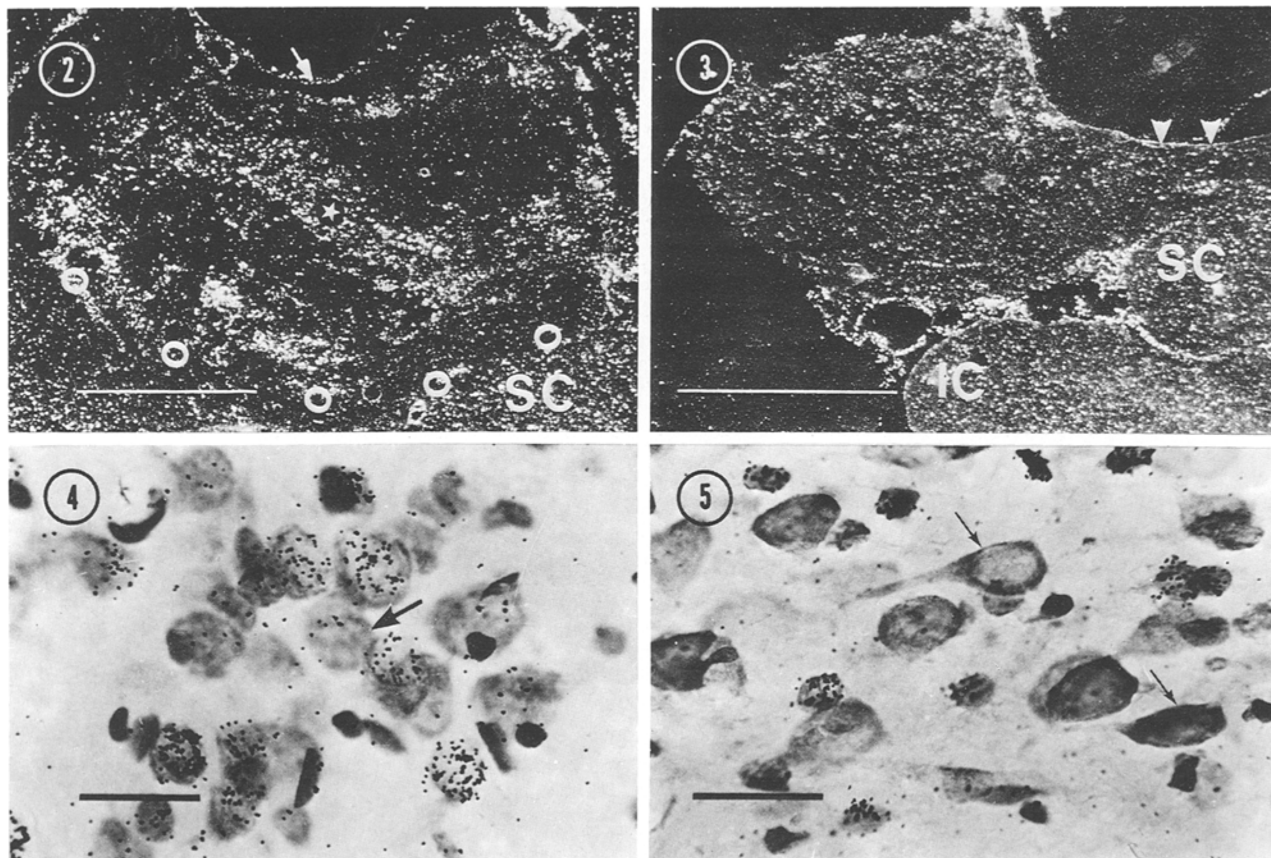

Fig. 2. Transverse section of cortical transplant (E17) labeled 3 days after transplantation. The transplant is located in the midline dorsal to the host superior colliculus (SC). See text for explanation. Scale 1 mm

Fig. 3. Parasagittal section of a mature cortical transplant (E18) connected by a fiber bundle (arrowheads) to the host superior colliculus (SC). Posterior is to the left. ^{3}HT was given 4 days after transplantation. See text for further description. IC – inferior colliculus; Scale 1 mm

Fig. 4. Labeled neurons and non-neurons in transplant shown in Fig. 2. Note neuron (arrow) labeled at background level adjacent to labeled neuron. Scale 25 µm

Fig. 5. Labeled non-neurons (glia cells) observed in transplant shown in Fig. 3. Neurons were not labeled. Note alignment of neurons (arrows) in an anterior (to the right) posterior direction. Scale 25 µm

ant number of labeled neurons (Table 2, Fig. 9). The numbers of neurons labeled declined, however, with increasing "age" of the graft. Only a few neurons were labeled in one of the E19 cortical transplants taken from a host injected with ^{3}HT on day 3 after transplantation. Neurons within transplants from E20 donors failed to take up ^{3}HT on day 2 post-transplantation. This corresponded to a similar failure of ^{3}HT uptake in neurons of E18 and E19 transplants on days 4 and 3 after transplantation, respectively (Table 2, Figs. 5, 11).

Non-neuronal cells were seen labeled in all the transplants studied. Distribution of non-neuronal cells labeled after termination of neurogenesis is shown in Fig. 3. In this example a transplant taken from an E18 donor was subjected to ^{3}HT 4 days after transplantation. Labeled non-neuronal cells occur relatively evenly dispersed throughout the transplant. Unlabeled neurons in this section are illustrated at higher magnification in Fig. 5.

Short survival studies show dense regions of labeled cells in the immature transplants subjected to ^{3}HT during the period of neurogenesis. Figure 6 shows an autoradiogram from a transverse section through the superior colliculus and the dorsally placed transplant from an experimental animal killed 1.5 days after birth. The cortical transplant was taken from an E16 donor and ^{3}HT was applied 1 day after transplantation. Labeled cells are seen in the ventral region of the transplant. Portions free of labeled cells

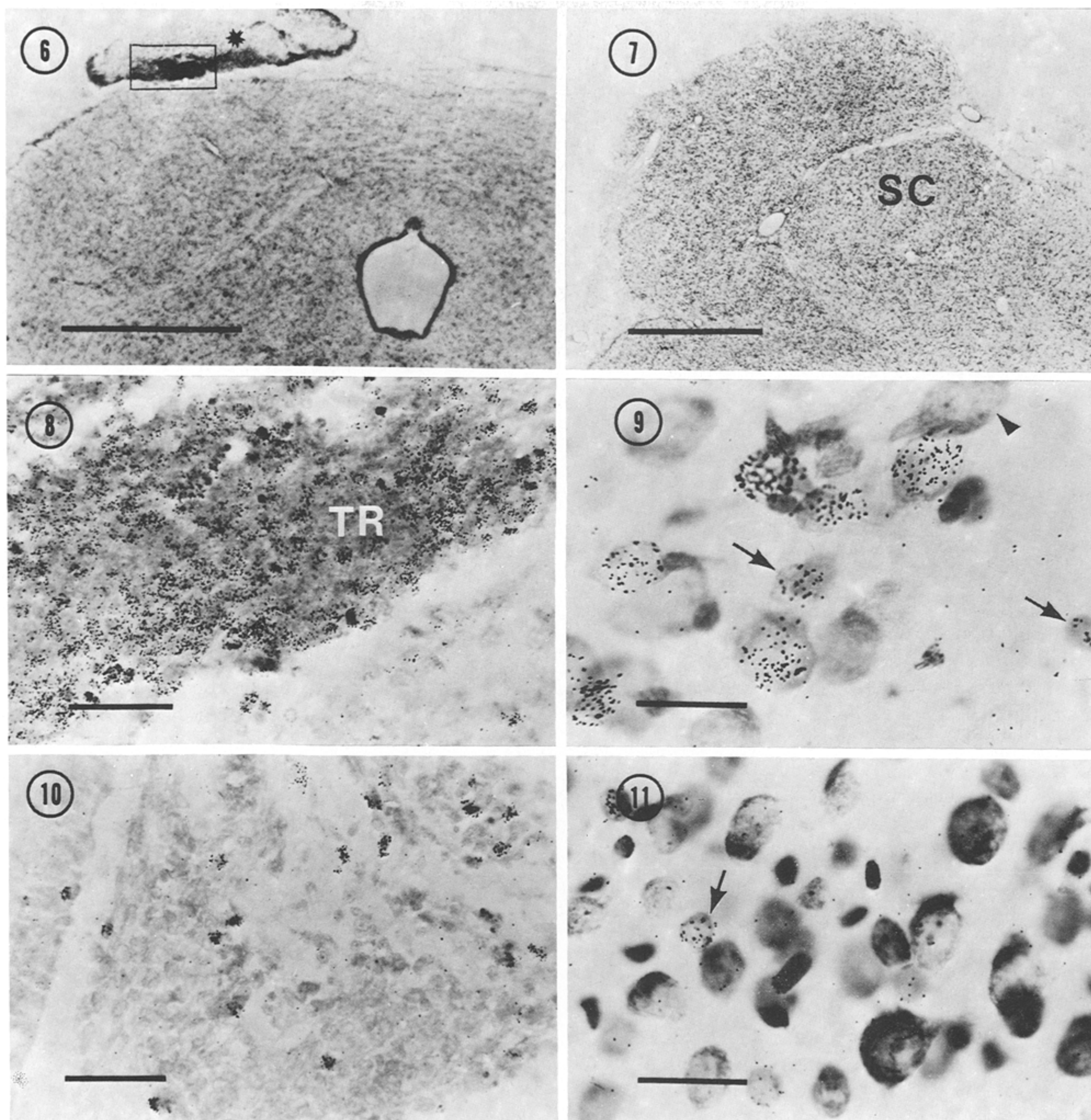

Fig. 6. Low power autoradiogram of a cross section through the midbrain of a 1.5 day old host. The implant from an E16 donor positioned dorsal to the left SC was subjected to ^{3}HT 1 day after transplantation and killed 6 h after labeling. Labeled cells were absent in the dorsal pale staining region of the transplant (*). Area in inset is shown at higher power in Fig. 8. Scale 1 mm

Fig. 7. Transverse section through a cortical transplant (7 weeks survival) positioned on the left side dorsal to the host superior colliculus (SC). The transplant was taken from an E19 donor and pulsed with ^{3}HT three days after transplantation. The observed labeling of non-neuronal cells is shown at higher power in Fig. 11. Scale 1 mm

Fig. 8. Autoradiogram showing labeling pattern of immature cells in transplant (TR) in the region outlined in Fig. 6. Large numbers of cells are labeled in the transplant. Scale 50 μm

Fig. 9. Labeled neurons and glia cells (arrows) are seen in an E19 cortical transplant that was labeled one day after transplantation (7 weeks survival). Note unlabeled neuron (arrowhead) adjacent to labeled neuron. Scale 25 μm

Fig. 10. Autoradiogram of immature transplant taken from an E19 donor, subjected to ^{3}HT 3 days after transplantation and killed 3 h after tracer injection. Note labeled cells are scattered throughout the transplant. Scale 50 μm

Fig. 11. Labeling of glia cells (arrow) in transplant shown at low power in Fig. 7. Scale 25 μm

(*) were present within immature transplants. At this early age of survival (1.5 days), no signs of tissue bridges between the transplant and the host brain were found. These bridges were commonly observed in mature transplants (Figs. 3, 7). A cortical transplant taken from an E19 donor that survived to maturity (7 weeks) in a similar position is illustrated in Fig. 7.

Cells labeled in the immature transplant shortly after transplantation (Fig. 8) may differentiate into neurons and non-neurons in the mature transplant (Fig. 9). Tracer applied after cessation of neurogenesis labeled relatively few cells scattered throughout the transplant (Fig. 10). Presumably all cells labeled at that time differentiated into non-neurons (Fig. 11).

Discussion

Studies show that neurons marked by silver grains are found in transplants which were labeled with ^{3}HT on fetal days E15–E18, 4–24 h before transplantation. This implies that cells which underwent their terminal division in the donor brain have survived the transplantation procedure. Since the majority of neurons in layers VI–IV and some in the upper layers of the cerebral cortex have been formed by E18 (Berry and Rogers 1965), we conclude that neurons expected to migrate to these layers have survived. Except for a few cases where a small number of non-neuronal cells generated within the transplant were found in the bordering host tissue, labeled cells remained in the confines of the transplant in all of our experimental animals. This is in contrast to the findings of Das and Altman (1971). These authors observed that transplanted cerebellar neurons become incorporated into the molecular and internal granular layers of the host cerebellum. Das and Altman (1971) used cerebellar grafts that were labeled with ^{3}HT on postnatal day 7 and subsequently transplanted (1 h after labeling) into the cerebellum of similar aged host rats. They proposed that the immature neurons migrated to their appropriate position in the host cerebellum. Although the mechanisms underlying neuron migration are little understood, it is clear from in vitro studies that the cell surface and the presence of certain macromolecules play a role in cell adhesion and motility (Trenkner and Sidman 1977). The quality and quantity of specific cell membrane components may change during the age of the neuron (Garber and Moscona 1972; Pfenninger and Bunge 1974; Hatten and Sidman 1976) influencing cellular interactions. In contrast to Das and Altman's study, the age of the transplant differed from that of the host tissue in our study. Thus, the failure of neuron migration from the transplant to the surrounding host brain could be explained on the basis of incompatibility of surface properties.

In addition to labeled neurons, there were also well-differentiated unlabeled neurons in the cortical transplants. The unlabeled neurons were derived from precursor cells that had their final mitoses either before or after transplantation. Exposing cortical grafts to ^{3}HT after placing them in the host brain, we observed that a number of precursor neurons will indeed continue to divide after transplantation.

With the notable exception of the olfactory epithelium (Graziadei and Graziadei 1978), neuron populations of the mammalian nervous system irrevocably lose their capacity to proliferate at a particular time in development. Exactly why this should be is uncertain. Several authors have suggested that environmental factors such as hormone levels (Balazs et al. 1971; Nicholson and Altman 1972; Lewis et al. 1976), nutrition (Patel et al. 1973) and particular drugs (Lewis et al. 1977; Patel et al. 1977) may have some effect in regulation of mitosis in the nervous system.

A further possibility is that the store of precursor cells becomes depleted as their progeny "differentiates". Holzer et al. (1975) propose that the daughter cells gain the capacity via a quantal mitosis step to synthesize a protein that is characteristic for the differentiated state only. A quantal mitosis may give rise to a differentiated cell and another precursor cell during an asymmetric division or, alternatively, to two differentiated cells during a symmetric division. In the latter event the precursor cells become used up and the cell population may lose its capacity for renewal. A recent study has shown that the final division of muscle precursor cells is a symmetric one giving rise to two differentiated cells that synthesize muscle specific creatine phosphokinase (Kligman and Nameroff 1980). Like differentiated muscle cells, neurons also lose their capacity to divide, and a similar mechanism may lead to neuron differentiation. Although features of specific biochemical differentiation remain to be established for the different neuron classes (Phelps and Pfeifer 1975; Nirenberg 1975; Varon 1977; Rothman et al. 1978), their mature forms can be recognized using morphological criteria. Studying early morphological differentiation of mouse retinal ganglion cells, Hinds and Hinds (1974) observed that the majority of ganglion cells appear to be derived from daughter cell pairs. This would suggest that neurons are, for the most part, formed like muscle cells by a symmetric final division.

In the present study, the transplanted precursor cells complete their "predetermined" program of histogenesis. This occurs independently of their position and the age discrepancy between host brain and transplant. In most cases neurons in the transplant stop proliferating at a time corresponding to that observed in the intact cerebral cortex, namely between E21 and E22 (Berry and Rogers 1965). A recent report shows mitosis of neuron precursors grown in aggregates in tissue culture (Sensenbrenner et al. 1980). Interestingly, as in the present study, the timing of neuron and non-neuron generation in the culture system was for the most part similar to that in vivo.

Mature cortical transplants contain neuron types that are characteristic of cerebral cortex (Das 1975, 1978; Jaeger and Lund 1978). However, their disposition within the cortical transplant differs from that of the intact cortex. The neurons generated in the transplant most commonly come to lie in discontinuous patches, while in the intact cortex they usually take up positions in a specific lamina. There are at least two explanations which could account for this. One is the inability of the young neurons to migrate properly, possibly as a result of mechanical disturbance of preestablished internal relationships, such as injury or misalignment of radial glia and cell death (or even lack of it). The second explanation relates to the influence of afferent fibers on the laminar distribution. Some cortical transplants may differentiate in the absence of extrinsic fiber connections while others receive a limited input of afferents from the host brain (Jaeger and Lund 1980). The effect of afferent fibers on developing target regions has been most extensively studied regarding the question of neuron survival, but some effect on developing laminar organization has been observed by Kelly and Cowan (1972) after deafferentation of the chick tectum. Disruption of laminar segregation could also result from extensive neuron death occurring as a result of the transplantation procedure. However, although some cells may die, it is clear that substantial numbers of neurons which underwent their final division prior to transplantation still survive.

To summarize, cortical transplants are composed of neurons generated before and after transplantation. Precursor cells continue mitosis following a schedule that essentially resembles normal neurogenesis. The cortical transplants may be useful for further studies of the conditions under which segregation into laminae occurs and neuron classes may exhibit their specific differentiated features.

Acknowledgments. The authors are pleased to thank Ms. Cindy Gue for her expert editorial assistance and typing of the manuscript. This research was supported by Grants EY-01950 and EY-03326 from the National Institutes of Health. The first author performed this study as partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biological Structure, University of Washington, Seattle, WA, USA.

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Received April 10, 1980