

Neuronal Maturation in an Experimental Model of Brain Tissue Heterotopia in the Lung

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Neural maturation involves diverse interaction and signaling mechanisms that are essential to the development of the nervous system. However, little is known about the development of neurons in heterotopic brain tissue in the lung, a rare abnormality observed in malformed babies and fetuses. The aim of this study was to identify the neurons and to investigate their maturation in experimental brain tissue heterotopia during fetal and neonatal periods. The fetuses from 24 pregnant female Swiss mice were used to induce brain tissue heterotopia on the 15th gestational day. Briefly, the brain of one fetus of each dam was extracted, disaggregated, and injected into the right hemithorax of siblings. Six of these fetuses with pulmonary brain tissue implantation were collected on the 18th gestational day (group E18), and six others were collected on the 8th postnatal day (group P8). The brain of each fetus from dams not submitted to any experimental procedure was collected on the 18th gestational day (group CE18) and on the 8th postnatal day (group CP8) to serve as a control for neuronal quantitation and maturation. Immunohistochemical staining of NeuN was used to assess neuron quantity and maturation. The NeuN labeling index was greater in the postnatal period than in the fetal period for the experimental and control groups ($P8 > E18$ and $CP8 > CE18$), although there were fewer neurons in experimental than in control groups ($P8 < CP8$ and $E18 < CE18$) ($P < 0.005$). These results indicate that fetal neuroblasts/neurons not only survive a dramatic event such as mechanical disaggregation, in the same way as it happens in human cases, but also they retain their development in heterotopia, irrespective of local tissue influences. *Exp Biol Med* 233:535–539, 2008

Key words: heterotopic tissue; experimental model; neuronal maturation; NeuN

Introduction

Central nervous system (CNS) development begins during the gastrula stage with the formation of the embryonic plates, during which important mechanisms involving cell interaction and signaling necessary for the formation of its different parts occur (1, 2). The CNS maturation sequence in rodents is similar to that in humans, although it occurs in days and is largely postnatal, whereas in our species it takes months and is mainly prenatal (3). However, little is known about neuronal maturation outside the CNS, such as that in brain tissue heterotopia in the lung, a rare abnormality observed in malformed babies and fetuses (4–7).

There are few studies regarding the mechanisms involved in brain tissue heterotopia in the lung and its implantation and neuronal development. Moreover, reports of human cases usually describe findings from autopsies of fetuses with severe cerebral lesions (8); thus, in these cases it is impossible to follow these cells after birth. This difficulty may be overcome by studying the experimental model of surgically induced brain tissue heterotopia in the lung described by Quemelo *et al.* in 2007 (9); this model allows further investigation of the mechanisms involved in cell interaction and maturation.

The main goal of the present study was to identify and quantify the presence and maturation of neurons in a mouse model of experimental brain tissue heterotopia in the lung during the fetal and neonatal periods by using immunohistochemistry (IH).

Material and Methods

Animals. The experiment was approved by the Ethics Committee for Animal Experimentation (CETEA) (process no. 018/2005).

Twenty-four female and six male Swiss mice were

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obtained from the colony of the Central Animal House of Ribeirão Preto Campus of the University of São Paulo. The animals received water and appropriate commercial mouse chow *ad libitum*. The facility in which the animals were housed throughout the experiment was equipped with sound protection, was rigorously controlled with 12:12-hr light: dark cycles, and had low luminosity, a constant temperature of 22°C, and an exhaust system for several changes of air during the day.

After a period of adaptation, the females were placed in daily contact with the males for mating and checked for a vaginal plug, the presence of which determined day zero of gestation. Pregnant females were kept in individual plastic cages until the end of the experiment.

Surgery was performed in the morning of the 15th gestational day (gd) under aseptic conditions, according to the method proposed by Quemelo *et al.* (9). Briefly, pregnant dams were anesthetized with 0.1 ml ketamine base solution (50 mg/ml) (Ketamina; Pfizer do Brasil Ltda, São Paulo, Brazil) and xylazine (10 mg/ml) (Rompum; Bayer do Brasil Ltda, São Paulo, Brazil) injected into the lateral muscles of the thigh; after the dams were anesthetized, median laparotomy was done to expose the uterine horns. One fetus was removed by hysterotomy, and its brain was removed, minced, disaggregated in RPMI culture medium (medium 1640 with L-glutamine and without sodium bicarbonate; Gibco BRL, Grand Island, NY), and introduced into the thorax of three siblings, with the aim of pulmonary brain tissue implantation (PBI).

Six fetuses with PBI were collected from different dams on the 18th gd (group E18), and six others were collected on the 8th postnatal day (pd; group P8). Fetuses with PBI collected by cesarean delivery during the gestational period were identified by their known positions in the uterus, whereas those from the 8th pd were identified after histologic analysis of all live-born fetuses. The same number of fetuses of control dams not submitted to any surgical procedures was collected on the 18th gd (group CE18) and on the 8th pd (CP8); the brain of each of these fetuses was used as a control for the neuronal quantitation and maturation.

Histology and IH. Immediately after collection, the trunks of fetuses with PBI and the heads of the controls were fixed in buffered formalin, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Four-micrometer histologic sections of each block were obtained for IH and hematoxylin and eosin staining.

Antigen retrieval was performed in a steamer with citrate buffer (pH 6.0) for 40 mins. IH using the polyclonal antibody anti-GFAP (1:500 in bovine serum albumin [BSA]; Dako Corp., Carpinteria, CA) was done with an immunoperoxidase method using the avidin-biotin complex (ABC). Secondary antibody (biotinylated, affinity-purified anti-immunoglobulin; Novocastra Laboratories, Newcastle upon Tyne, UK) was incubated for 30 mins and then further incubated with the ABC universal kit (Novostain Super ABC Kit, universal; Novocastra Laboratories) also for 30

mins. IH using the monoclonal antibody anti-NeuN (1:1500 in BSA; Chemicon, Temecula, CA) was performed with SuperPicTure Polymer Detection Kit (Zymed Laboratories; Invitrogen, San Francisco, CA) for 30 mins. The DAB substrate was used for the development of the immunohistochemical reaction (D5638; Sigma, St. Louis, MO) for 10 mins; subsequently counterstaining was done with hematoxylin for 10 secs. All analyses were carried out by using a standard light microscope.

Data Analysis. The location and the morphologic and immunohistochemical characteristics of heterotopic brain tissue implants were analyzed. Only cells with nuclear staining by anti-NeuN antibody of all groups were considered positive and were counted by using a 40× objective lens. The labeling index (LI) for NeuN was obtained by dividing the number of positive cells by the sum of positive and negative ones. All cells of all heterotopic brain tissue implants from groups E18 and P8 were counted and compared to the mean NeuN count from six areas chosen at random from the mesencephalon and telencephalon regions of groups CE18 and CP8.

Data were analyzed statistically by the Mann-Whitney test using the GraphPad Prism 4.00 software (Prism, Chicago, IL), with the level of significance set at $P \leq 0.05$.

Results

Only 15 (62.5%) of the 24 females initially assigned to the experiment could be used. Five (20.8%) died of anesthetic complications, and four (16.6%) aborted on the 16th gd.

Microscopic examination of the fetal trunk slides stained with hematoxylin and eosin showed that fetuses of the E18 and P8 groups presented brain tissue fragments, which consisted of small cells with dense ovoid nuclei and scarce cytoplasm, arranged in plaques on a fibrillar background (Fig. 1A and B). These fragments of brain tissue were often implanted in parietal and visceral pleura as well as in the pulmonary parenchyma. The brain tissue, which was positive for GFAP and NeuN, was well preserved, showed mitoses (Fig. 1E), was vascularized, and was usually adjacent to blood vessels.

The heterotopic neurons immunostained with anti-NeuN antibody in groups E18 (Fig. 1E) and P8 (Fig. 1F) were surrounded by abundant glial cells (Fig. 1C and D). The NeuN LI was greater in heterotopic brain tissue in the P8 group than in the E18 group ($P < 0.05$). The NeuN LI in the brain tissue in the CP8 group was greater than that in the CE18 group ($P < 0.05$). However, there were more neurons in the brain tissue of the control groups CE18 and CP8 than in the corresponding heterotopic brain tissue in the groups E18 and P8, respectively ($P < 0.05$; Table 1).

Discussion

Little is known about heterotopic brain tissue implantation, proliferation, and interaction in the lung, mainly

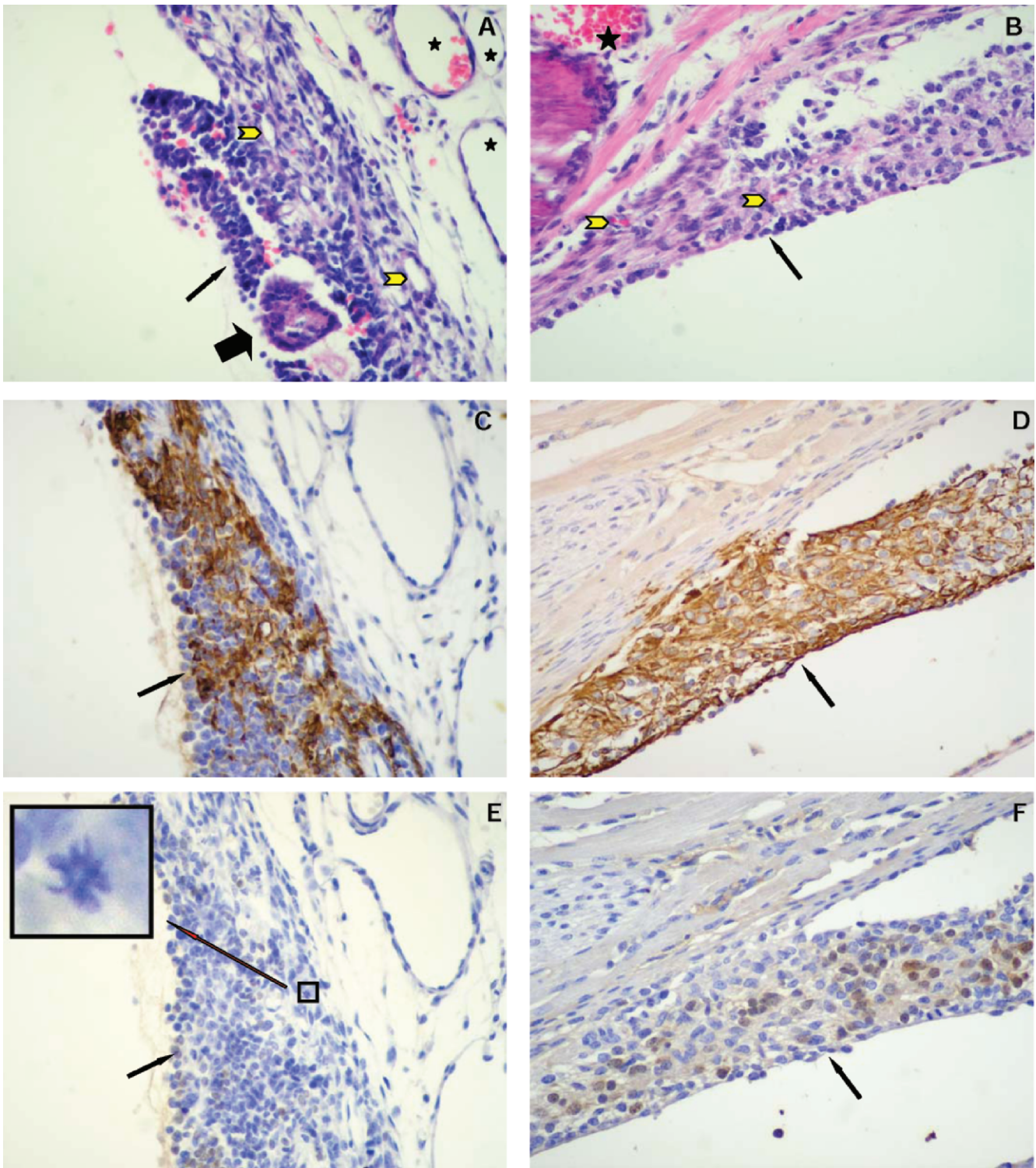


Figure 1. Photomicrographs of histologic sections of the fetal trunks with heterotopic brain tissue. (A, C, and E) Tissues are from members of group E18. (B, D, and F) Tissues are from members of group P8. (A) Shown is a focus of heterotopic brain tissue implanted on parietal pleura (small arrow). The small round cells with scant cytoplasm are arranged in sheets and sometimes exhibit a rosette formation (large arrow). These cells are on a fibrillary background containing blood vessels (arrow head). Larger vessels are observed in the mesenchymal tissue of the host (asterisks). The stain was hematoxylin and eosin. (B) The implanted brain tissue is better differentiated, with cells presenting larger nuclei and resting in an abundant fibrillary background. Capillaries containing red blood cells are seen inside the implanted tissue (arrowhead), which is near larger blood vessels of the host (asterisk). The stain was hematoxylin and eosin. (C and D) The immunohistochemical reaction was achieved by using the ABC method with the primary anti-NeuN antibody and the developing agent DAB. There are fewer GFAP-positive and NeuN-positive cells in heterotopic brain tissue in group E18 (C, E) than in group P8 (D, F); this difference indicates maturation of the implanted tissue. (E) Mitosis in fetal heterotopic brain tissue is highlighted. Original magnification: $\times 400$.

Table 1. NeuN LI in Fetal and Neonatal Control Brain and in Fetal and Neonatal Heterotopic Brain in the Lung

	Control brain group		PBI group		<i>P</i> value
	CE18	CP8	E18	P8	
NeuN LI	38%	59%	4%	22%	0.05

because only recently was an experimental model developed (9) that will allow the investigation of the mechanisms involved in this abnormality, including those seen in human cases. Despite rarity of heterotopic brain tissue implantation and the lack of immediate practical application, the understanding of the biological phenomenon involved in the implantation, interaction, and survival of the brain tissue in a place different from its origin is relevant, because some of the most important aspects of the life of a neuron depend upon cell-cell as well as cell-extracellular interaction and cell signaling.

Although brain tissue heterotopia is self-limited because anencephalic fetuses are aborted or die shortly after birth, a concern has recently emerged in cases of multiple organ transplantation of lungs and heart. Donors are frequently those who have major brain injury due to car accidents or falls, and in such events, emboli may originate from fragments of brain tissue and may lodge in the lung circulation, where they may grow; in such cases the understanding of brain tissue heterotopia in the lung would be useful.

In the present study, the experimental model was efficient in inducing brain tissue heterotopia, and development of this tissue continued on the 8th pd. The implanted fragments were well preserved, adhering to the parietal and visceral pleura or even locating inside the lung parenchyma. The presented blood vessels contained red blood cells and few or no inflammatory cells. The viability of the injected fragments was demonstrated by the scarcity of necrosis, hemorrhage, degeneration, and inflammation and was confirmed by the presence of mitoses.

The heterotopic brain tissue presented fewer cells immunostained with anti-NeuN antibody than with anti-GFAP in the fetal and postnatal periods, and this result is in accordance with the findings in human cases reported by Kanbour *et al.* (4) and Chen *et al.* (5), who identified fewer pyramidal neurons among the more common glial cells. This finding was interpreted in light of the notion that there is a 10:1 ratio of glial cells to neurons in the CNS (10, 11). However, when we compared the heterotopic brain tissue with the control brain, we found that there were fewer neurons in the former than in the latter on the same day, whether during the fetal or perinatal period. This finding indicated that there was a selective reduction in the neural population. One possible explanation is that the immature or mature neurons were more likely to be injured than were the

glial cells (12) and were more affected by the mechanical or biochemical actions or anoxia induced by the procedures involved in this method. It is reasonable to suppose that the extraction of the brain of the donor fetus, its mechanical disaggregation, and final injection into the thorax of the receptor siblings is quite aggressive and potentially induces the death of more specialized cells. A similar mechanism possibly occurs in human cases, because one explanation is that the disruption of the exposed brain tissue in neural tube defects results in the aspiration of floating brain fragments in the amniotic fluid that eventually lodge in the lung (6, 7, 13).

Although the ratio of neurons to glial cells in both experimental groups was decreased in respect to the control brain during the same prenatal or postnatal period, the NeuN LI was greater in postnatal than in prenatal groups, whether experimental or control; this finding indicated that neuronal maturation was not halted in the heterotopic brain tissue. Neurogenesis in mice is mainly postnatal (3); thus, the regulation of neural development in the heterotopic brain tissue was preserved, at least in part, despite the possible microenvironmental influences of the lung tissue.

The increased ratio of neurons to glial cells observed in the postnatal period may also reflect the immunoreactivity of NeuN in neurons, which begins on gd 9.5 but is more intense in the terminally differentiated cells; this difference suggests that neurons become detected by this method in a later or postmitotic period (14). This finding was similar in control and experimental groups, and this similarity showed that the heterotopic brain tissue retained the ability to undergo neuronal maturation. An additional way of neuron generation is from subventricular astrocytes, which therefore act as a type of stem cell (15, 16). Neurogenesis is highly restricted to certain areas of the adult mammal (17–21), whereas glial cells proliferate in many regions of throughout life (22).

Although we do not know the exact origin of the fragments, which show a different composition depending on the stage of development, or the number of viable cells that were injected, it is assumed that the mechanical disaggregation of the brain renders bits of tissue rather than isolated cells. These small bits of brain come from different regions, but they retain their structure and spatial distribution; therefore, it was necessary to know the average NeuN LI cells in the brain of controls for comparison. It was the proportion of NeuN-labeled cells in control and implanted brain tissue that was important, because in both cases there was a progressive increase of these cellular elements over time. However, the present study showed that neuronal development was seen, and future studies may address this question by isolating specific areas such as the cerebellum, mesencephalon, and subventricular and cortical regions to understand the mechanisms of interactions between the lung and brain tissue.

In conclusion, our results showed that the NeuN LI was greater in the P8 group than in the E18 group, demonstrating

that the process of neuronal maturation occurs in the heterotopic brain tissue in the lung in the same way as in the brain. However, many aspects of cell interaction and signaling related to the process of neuronal maturation as well as the mechanisms of implantation, proliferation, and fate of brain tissue heterotopia need to be further investigated.

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