Expression of Nervous Tissue Nuclear Protein and Glial Fibrillary Acidic Protein during Morphogenesis of the Neocortex

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We studied the distribution of glial fibrillary acidic protein (GFAP) and neuron-specific nuclear histone protein NeuN in sulci of the brain cortex during the pre- and postnatal ontogeny. The expression of GFAP during morphogenetic development of the sulci and gyri is cyclic. After functional reorientation of GFAP in human fetuses at weeks 28-30 it ceases to be a marker of morphogenetically active glia and becomes a marker of glial cells exclusively. Redistribution of NeuN expression in different layers of sulci during their formation was found: enhanced expression of NeuN in the cortical layer 6 of sulci and its reduced expression in the upper layers were noted, whereas outside the cortical sulci NeuN expression was similar in all layers. At weeks 24-25 of gestation, NeuN serves as a marker of ingrowth of secondary visual fibers from the dorsal thalamus.

Key Words: *calcarine fissure; neuroblast; NeuN; secondary visual fibers*

Study of the mechanisms of sulcus and girys formation in mammalian girifyed brain remains one of the most important problems of modern perinatology. It is known that division of neuroblasts occurs in proliferative zones, from which they migrate into the forming cortical plate. Glutamatergic neurons migrate radially from the ventricular zone along glial fibers labeled with glial fibrillary acidic protein (GFAP) [3,4]. GABAergic interneurons are formed in the ganglionic tubercles and then tangentially migrate into the developing cortical plate [5]. An important role in the developing cortex is played by afferent fibers synaptically cooperating with neurons [2]. Neuron-specific nuclear histone protein NeuN, which serves as neuronal marker in adults, manifests episodically in human fetuses [6]. It is present in the ventricular, subventricular, and partially cortical zone neuroblasts from week 19 and disappears after week 24 of human prenatal development [6].

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We traced the regularities of sulcus development in human fetuses using two markers, GFAP and NeuN.

MATERIALS AND METHODS

The study was carried out on autopsy material. Specimens of the brain hemispheres from 17 fetuses of weeks 14-40, one infant aged 10 months, and 2 adults were examined. The material was fixed in 10% formalin and Bouin fixative. Specimens of the medial hemispheric wall of the occipital lobe containing the calcarine and parieto-occipital sulci were processed by standard histological methods and embedded in paraffin. The sections (10 μ) were stained by the Nissl method.

Immunohistochemical staining with anti-GFAP Ab-1 mouse (Lab Vision) and anti-NeuN mouse (Chemicon) antibodies was carried out. UltraVision Plus Detection System (Lab Vision) was used for visualization. The concentrations (*C*) of labeled cells were evaluated by Image Scope M software:

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$$C=n/S,$$

where n is cell count and S is the size of area of interest. Statistical significance of differences was verified by Fisher's F test using Test 1.0 software.

RESULTS

Our previous data indicate that the medial hemispheric wall has primary sulci, the folds of the entire hemispheric wall, from week 14 [1]. Radial glial processes guiding neuroblasts migration are seen in the frontal sections of fetal (14 weeks) brain stained by the Nissl method. No expression of GFAP and NeuN was detected in them.

The expression of GFAP in the radial glia was detected on week 16, which suggests the onset of some morphogenetic events. Cell bodies are located in the ventricular zone, while their processes are aimed radially towards the marginal zone. Chains of neuroblasts were found along the GFAP-positive ($GFAP^+$) fibers of the radial glia. At the bottom of the primary sulci, $GFAP^+$ fibers separate tangentially, but not vertically, as in hemispheric walls without sulci. Tangential division of $GFAP^+$ fibers is less pronounced under the parieto-occipital sulcus than under the calcarine fissure. NeuN expression starts from week 16. NeuN-positive ($NeuN^+$) cells are found in the subventricular, intermedial, and cortical zones of the hemispheric wall. The concentration of $NeuN^+$ cells in the ground floor of the cortex is slightly higher (Table 1). No differences in the distribution of $NeuN^+$ cells in the calcarine and parieto-occipital sulci were seen. According to published data, this marker appears in human fetal cortex only from week 19 [6].

The age of 18-20 weeks is characterized by straightening of the primary parieto-occipital sulcus, while the calcarine fissure is well expressed [1]. The bodies of $GFAP^+$ cells are located in the ventricular and subventricular zones of the hemispheric wall. The count of $GFAP^+$ cells in the hemispheric medial wall is significantly lower in the subventricular than in the dorsal zone, and their location is largely associated with the subventricular zone. The expression of GFAP in the neocortex is in general reduced. The distribution of $NeuN^+$ cells in the dorsal neocortex is even (7.59×10^{-3} cell/μ^2), while in the medial neocortex various redistributions of this marker in the cortical layers are observed. The $NeuN^+$ cells at the bottom of the calcarine fissure are concentrated mainly in layer 6 (3.61×10^{-3} cell/μ^2), while the upper layers contain virtually no stained cells (1.62×10^{-3} cell/μ^2; $p<0.05$). Uniform distribution of $NeuN^+$ cells in the cortical plate is seen at the site of straightened parieto-occipital sulcus (Table 1). Solitary $NeuN^+$ cells are also found in the subventricular and intermedial zones of the hemispheric wall.

Both studied sulci were again clearly seen at weeks 21-23. An appreciable level of $GFAP^+$ cells

TABLE 1. Concentration of $NeuN^+$ Cells ($\times10^{-3}$ cell/μ^2) in Cerebral Occipital Cortex during Human Ontogeny

Age group	Prenatal ontogeny	Calcarine fissure		Parieto-occipital sulcus	
		layers 2+3	layers 4+5+6	layers 2+3	layers 4+5+6
Cortex outside sulci (layers 2+3+4+5+6)					
weeks 16-17	3.19	7.34	3.61	8.05	8.22
weeks 18-20	1.62	3.12	1.92	2.13	7.59
weeks 21-23	5.89	9.29	6.26	6.05	8.02
weeks 24-25	0.675	4.305	0.146	0.504	0.412
weeks 26-27	0.925	3.41	0.293	1.35	2.80
weeks 28-29	0.93	6.75	0.801	3.47	4.30
weeks 30-31	0.738	5.57	0.272	0.98	3.60
weeks 32-33	0.187	4.98	0.031	0.173	2.09
weeks 34-35	0.0705	1.58	0.684	1.24	2.94
weeks 36-41	0.0617	0.541	0.212	0.00203	1.52
Postnatal ontogeny					
10 months 1 week	0.00741	0.254	0.00802	0.235	0.497
39-48 years	0.97	1.05	0.753	1.42	1.07

was found in the hemispheric medial and dorsal walls during this period. The direction of fibers under the sulci was the same as before (tangential). The count of $GFAP^+$ cells was lower under the calcarine fissure, presumably because this area is the thinnest in the entire hemispheric wall. The $NeuN^+$ cells were found in the entire cortex, their expression being more intense in layer 6. A significant ($p<0.05$) decrease in the count of $NeuN^+$ cells was found in the upper layer of the cortex (Table 1). Solitary $NeuN^+$ cells were found in the subventricular and intermedial zones.

On weeks 24-25, the calcarine fissure straightened, while the parieto-occipital sulcus remained intact. The count of $GFAP^+$ cells decreased compared to the previous stage. $GFAP^+$ fibers separated tangentially under the parieto-occipital sulcus and radially under the calcarine fissure. Hence, the expression of GFAP was cyclic, it decreased during the period when one of the studied sulci is absent. $NeuN^+$ cells were detected only in the cortical plate. On the whole, a drastic decrease in the level of this marker was observed up to its complete disappearance in many neocortical regions (which is in line with published data [6]). Solitary stained cells in the parieto-occipital area were found in the deepest layer of the cortex. Solitary $NeuN^+$ cells were found in all layers of the cortex in the area of its bending towards the calcarine fissure. Their presence was obvious at the site of the prospective calcarine fissure ($p<0.01$) in layer 4 (7.36×10^{-3} cell/μ^2) and less so in the deeper layers 5 and 6 (1.25×10^{-3} cell/μ^2) and upper layer 3 (1.73×10^{-3} cell/μ^2). Hence, the expression of NeuN protein in the visual cortex was heterogeneous; it was actively expressed in the prospective visual field 17, though before the beginning of cytoarchitectonic differentiation of the cortex. The outer geniculate body fibroblasts were differentiated during this period and the afferent fibers grew into the primary visual field 17 [2,4]. The interface of their penetration was presumably labeled by hyperexpression of NeuN in layer 4.

At weeks 26-27, two sulci are expressed, though the calcarine fissure is less deep. The level of $GFAP^+$ is again high at this stage. The direction of the fibers under the sulci is the same as previously: they diverge tangentially, while outside the sulci they diverge radially. $NeuN^+$ cells are found only in the cortical plate. These cells are just solitary in the parieto-occipital area and are located in the ground floor of the cortex. Solitary $NeuN^+$ cells are found in the entire thickness of the cortex at the site of its bending towards the calcarine fissure. In the calcarine fissure, they are located ($p<0.05$) in layer 4 (5.1×10^{-3} cell/μ^2) and less so in the deeper layers 5 and 6 (1.72×10^{-3} cell/μ^2) and upper layer 3 (0.82×10^{-3} cell/μ^2). The distribution of $NeuN^+$ cells virtually does not differ from that at the previous stage.

At weeks 28-29, both studied sulci are clearly seen. $GFAP^+$ cells appear in the cortical marginal layer. Many $GFAP^+$ cells whit retractred processes are present in the intermedial zone. It seems that GFAP is not longer a marker of morphogenetically active radial glia, but becomes a marker of glial cell differentiation. The distribution of NeuN outside the neocortical sulci is in general even, though in some places its level is slightly higher in the ground floor of the cortex. Marked redistribution of the staining is seen in the sulci: the lower cortical layers are stained more intensely, while the upper layers at the bottom of the sulci contain lesser numbers of stained cells. Immunohistochemical reaction to NeuN in the lower layers 5 and 6 of the calcarine fissure is much more intense than in the same layers outside the sulci. Less intense staining of the upper layers is found in the parieto-occipital sulcus, though the difference is less pronounced than in the calcarine fissure (Table 1).

Both studied sulci are expressed at weeks 30-35. Distribution of GFAP is similar to that during the previous stage. $GFAP^+$ cells are found in the ventricular, subventricular, intermedial zones and in the neocortical marginal layer, in which their processes form finely fibrous "cotton" in the sublying cortex. $NeuN^+$ cells are found in all layers on the brain surface. The incidence of $NeuN^+$ cells decreases significantly in the depth of the sulci from layer 2 to layer 6 of the cortex ($p<0.05$). The expression of NeuN decreases in both sulci with maturation of the fetuses. At 30 weeks, $NeuN^+$ cells are found at the bottom of the calcarine fissure in layers 6, 5, and partially 4, while at 35 weeks they are retained mainly in layer 6 of the calcarine fissure bottom (Table 1; Fig. 1). It is noteworthy that layers 5 and 6 of the calcarine fissure bottom are stained more intensely than the same layers in the cortex of smooth areas (gyri). Redistribution of stain was also seen in the parieto-occipital sulcus, but the counts of positive cells were significantly lower than in the calcarine fissure, and there were almost no stained neurons at the bottom. A drastic redistribution of $NeuN^+$ cells is found in tertiary sulci about 1 mm deep in field 17: the expression of NeuN sharply decreases in the upper layer and predominates in layer 4. Tertiary sulci in field 18 lost $NeuN^+$ cells in the entire thickness of the cortex.

At weeks 36-41, the distribution of GFAP was similar to that during the previous stage. $NeuN^+$ cells were found only on the brain surface, their content significantly decreased in the sulci, with just solitary cells found at the bottom of both sulci.

GFAP immunoreactivity at the age of 10 months of life was the same. $NeuN^+$ cells were present in all layers of the cortex, but only on the brain surface. Their levels decreased in the walls of both sulci, with

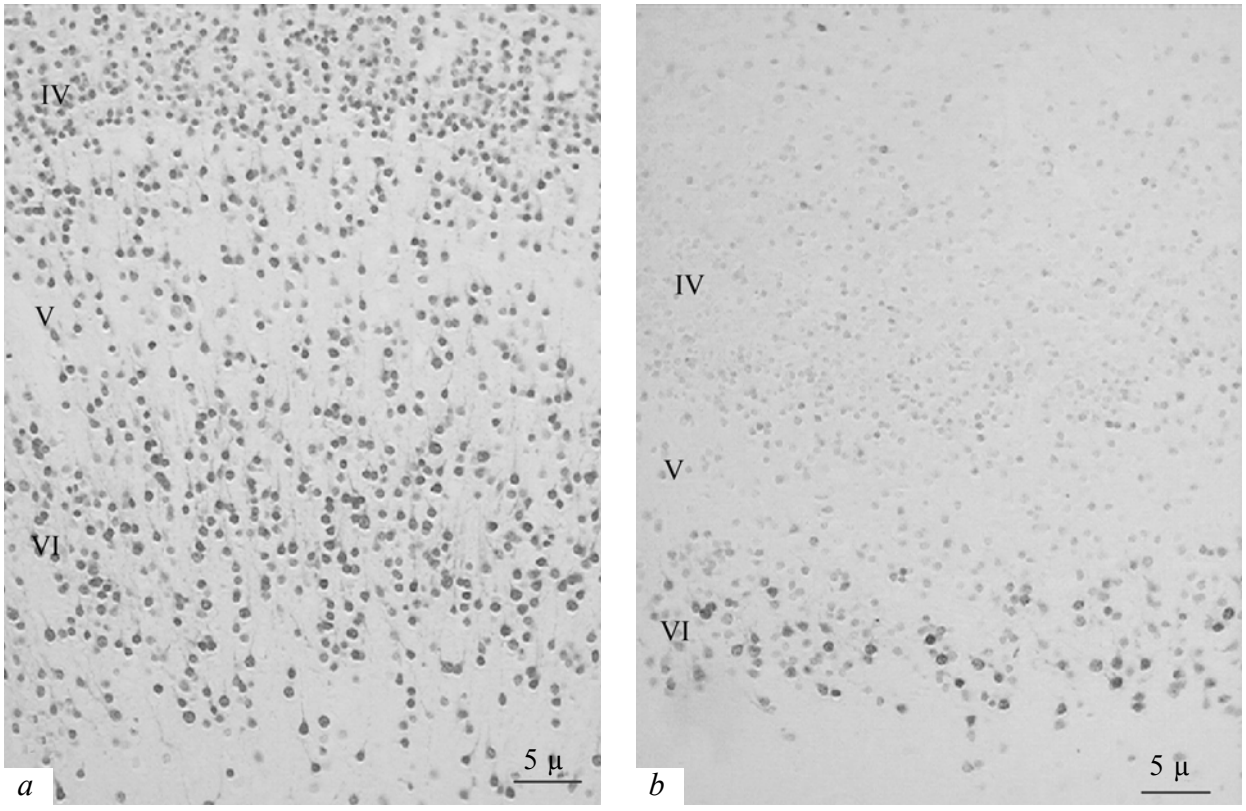

Fig. 1. Human fetal brain (35 weeks). Neocortical field 17. Immunohistochemical staining for NeuN, ×200. *a*) girys area; *b*) calcarine fissure bottom. Figures show the neocortical layers.

just solitary cells at the bottom (Table 1). The counts of $NeuN^+$ cells decreased in the tertiary (2-3 mm deep) sulci, but not so sharply as in deep sulci. Remarkably, protein location in the neuron started to resemble that in adults: it was located not only in the nucleus, but also in the cytoplasm delineating the axonal morphology at the neuron body.

In adults aged 38-49 years, GFAP is a marker of astrocytes, numerous in the hemispheric wall. $GFAP^+$ astrocytes in the marginal layer are worthy of note: a dense cotton-like network of axons stretches from them into the cortex, reaching cortical layer 5. The direction of the axons is different in the cytoarchitectonic fields. They are directed radially in field 17 and tangentially in the adjacent fields 18 and 19. $NeuN^+$ neurons are present in all studied sulci and giry of the occipital medial area and in all neocortical layers in adults. The neuron staining intensity is the same. Comparing the slices stained by the Nissl method we can say with high probability that all hemispheric neurons are stained.

Hence, the expression of NeuN in neocortical sulci and outside them is asynchronous up to the age of 10 months of postnatal development. It remains unclear at what age the expression of NeuN acquires an adult pattern.

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