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Postnatal Morphogenesis of Purkinje Cells in the Rat Cerebellum

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Electron microscopic and histochemical methods were used to obtain a quantitative assessment of the development of organelles in Purkinje cells in the cerebellum of mongrel rats ($n = 36$) during the postnatal period of ontogeny (days 2, 7, 15, and 45). The nucleus:cytoplasm ratio decreased, while nucleolar area increased, the size and length of mitochondria increased, the quantity of membrane-bound ribosomes and the length of endoplasmic reticular channels increased, the number and sizes of lysosomes increased, and the relative area occupied by lysosomes on sections of the cytoplasm also increased.

Keywords: cerebellum, Purkinje cells, ultrastructure, morphometry.

The formation of the definitive structure of the cerebellar cortex in rats occurs over a prolonged period of postnatal ontogeny [7]. This process is linked with neuron differentiation, the formation of neural connections, and their functional specialization [6]. Purkinje cells (PC) in the cerebellum receive information arriving in the cortex and send it along their axons. Data on the number, structure, and ultrastructural organization of these neurons are needed for understanding cerebellar morphogenesis [8].

In rats, the precursors of PC form on days 13–16 of embryogenesis in the ventricular layer of the roof of the fourth ventricle and then migrate in the radial direction, forming a multilaminar layer, the PC layer becoming unilaminar only by days 3–4 of postnatal development. Alignment of PC into a monolayer is mediated by mechanical factors: the pressure of the thickening of the granular layer and the barrier formed by parallel fibers, preventing displacement of PC perikarya into the molecular layer. Then, by age 12 days, the distance between PC perikarya increases rapidly, which is linked with the formation of the neuropil around them. Increases in the sizes of the perikarya themselves and dendrite growth continue to day 30 and are accompanied by ultrastructural changes in these neurons [5]. In neonatal rat pups, the PC cytoplasm contains large numbers of free ribosomes, forming Golgi complexes and, by day 10, large numbers of mitochondria and extensive smooth endoplasmic reticulum; PC bodies are surrounded by glial cells [4]. By age 15 days, the number of membrane-bound ribosomes increases [5]. Postnatal differentiation is seen at the light microscope level, with increases in perikaryon size, decreases in the number per unit length of gyrus, and changes in the cytoplasmic chromatophilia ratio of these cells [2]. The literature contains no quantitative assessments of the development of PC organelles in the phylogenetically ancient cerebellar cortex in rats during postnatal ontogeny.

The aim of the present work was to carry out a qualitative and quantitative assessment of the dynamics of postnatal organellogenesis in developing PC in the rat cerebellum.

Materials and Methods. Experiments were performed on female mongrel white rats with a starting weight of 180 ± ± 20 g and their offspring – 36 rat pups (12 for electron microscope studies and 24 for histochemical studies). Studies were performed in compliance with the "Regulations for Studies Using Experimental Animals" and were approved by the biomedical ethics committee of Grodno State Medical University (protocol No. 7 of December 23, 2013). Animals were kept on a standard animal-house diet. A single rat pup was collected from each female on reaching ages 2, 7, 15, and 45 days; animals were decapitated. The characteristics of PC metabolism were studied after freezing cere-

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TABLE 1. Sizes of Cerebellar Purkinje Cells (PC) at Different Time Points in the Postnatal Development of Rats (Me ± IQR)

Item	Indicator	Age of rats, days			
		2	7	15	45
PC perikaryon	Area, μm²	48.39 ± 4.27	94.68 ± 26.25*	189.97 ± 26.45*#	190.95 ± 18.57*#
PC nucleus	Area, μm²	25.61 ± 3.38	37.15 ± 9.95*	56.13 ± 9.17*#	48.61 ± 13.22*#+
PC nucleolus	Area, μm²	0.86 ± 0.34	1.97 ± 0.76*	3.04 ± 0.64*#	3.61 ± 0.50*#
PC	Nucleus:cytoplasm ratio	1.05 ± 0.49	0.78 ± 0.22*	0.46 ± 0.03*#	0.32 ± 0.07*#

Here and Table 2: *significant differences compared with values on day 2, #compared with values on day 7, +compared with values on day 15, $p < 0.05$.

bellum samples in liquid nitrogen. A Leica CM 1850 cryostat ((Leica Microsystems GmbH, Germany) at –14°C was used to cut sagittal cryostat sections, which were processed to detect succinate dehydrogenase activity (SDH, succinate:acceptor oxidoreductase).

The locations of the phylogenetically ancient parts of the cerebellar cortex in histological specimens in the developing cerebellum in rat pups were identified as described by Olenev [3] and Paxinos and Watson [11]. Examination of histological sections, microphotography, and morphometric studies were performed using an Axioscop 2 Plus microscope (Zeiss, Germany) and a digital video camera (Leica, DFC 320, Germany), along with ImageWarp image analysis software (Bitflow, USA). Quantitative assessment of SDH activity was performed by measuring the optical density of the chromogen reaction precipitate in the neuron cytoplasm at the absorption peak of the colored reaction product. Relative enzyme activity was expressed in optical density units.

Electron microscopic studies were performed by immersing cerebellar fragments in 1% osmium fixative in Millonig buffer pH 7.4. for 2 h at 4°C [10]. Specimens were then washed with a mixture of Millonig buffer (20 ml) and sucrose (900 mg), dehydrated in ascending ethanol concentrations and acetone, passed through a mixture of resins (Araldite M + Araldite M hardener 964 + dibutylphthalate + Araldite M accelerator 960) (Sigma-Aldrich) and acetone, and embedded in casting resin. Ultrathin sections of thickness about 35 nm were then cut on an EM UC 7 ultramicrotome (Leica, Germany), loaded onto copper support grids (Sigma, grid size 300 × 83), and contrasted with uranyl acetate and lead citrate [12]. Preparations were examined in a JEM-1011 electron microscope (JEOL, Japan) and photographed with an Olympus MegaView III digital camera (Olympus Soft Imaging Solutions, Germany). Ultrastructural morphometry was performed using iTEM image analysis software (JEOL, Japan). The areas of PC perikarya, nuclei, and nucleoli were measured, the nucleus:cytoplasm ratio was determined, the number of lysosomes was counted and their individual and relative areas in the cytoplasm were determined; mitochondrial shape was identified and the number and lengths of cristae per μm² of mitochondria were determined, along with the widths of Golgi complex (GC) cisterns, the lengths of rough endoplasmic reticulum (RER) cisterns, and the number of bound ribosomes per 1 μm of RER cistern length. The resulting mean values from animals of each experimental group were analyzed by nonparametric statistics in Statistica 6.0 for Windows (StatSoft Inc., USA). The descriptive statistics for each parameter consisted of the median (Me) and interquartile range (IQR).

Results. During the postnatal period of ontogeny in rats (from day 2 to day 15), differentiation was accompanied by progressive increases in the sizes of PC perikarya and nuclei. By day 45, perikaryon size had ceased to increase and neurons even decreased in size. There was a three-fold decrease in the nuclear-cytoplasmic ratio. PC nucleoli increased in size from day 2 to day 45. On day 45 after birth, area was four times greater than on day 2 (Table 1).

In rat pups aged two days, PC were distributed in several rows and all were normochromic. PC nuclei were oval, the nuclear envelope formed shallow invaginations, and the perinuclear space was narrow. Nuclei contained euchromatin, which was gently condensed at the nuclear envelope. Nuclei contained one central or several eccentrically distributed nucleoli, consisting of fibrillar and granular components. A large proportion of the cytoplasm was concentrated in the apical part of PC, where mitochondria, mostly round in shape, were distributed uniformly (Fig. 1, *a*).

Some mitochondria had electron-dense matrix. RER cisterns were short (see Fig. 1, *e*) and were located alongside mitochondria. GC were not yet completely formed, but generally consisted of groups of tightly appressed vacuoles or, more rarely, several short cisterns parallel to each other (see Fig. 1, *i*). The forming RER consisted of short channels. Only free ribosomes were present in the basal part of the perikaryon.

On day 2 of the postnatal period of ontogeny, the number of mitochondria per unit area of cytoplasm was maximal; the number decreased twofold by day 45 as the perikaryon enlarged, though size increased progressively (days 2–45). The area occupied by mitochondria in the PC cyto-

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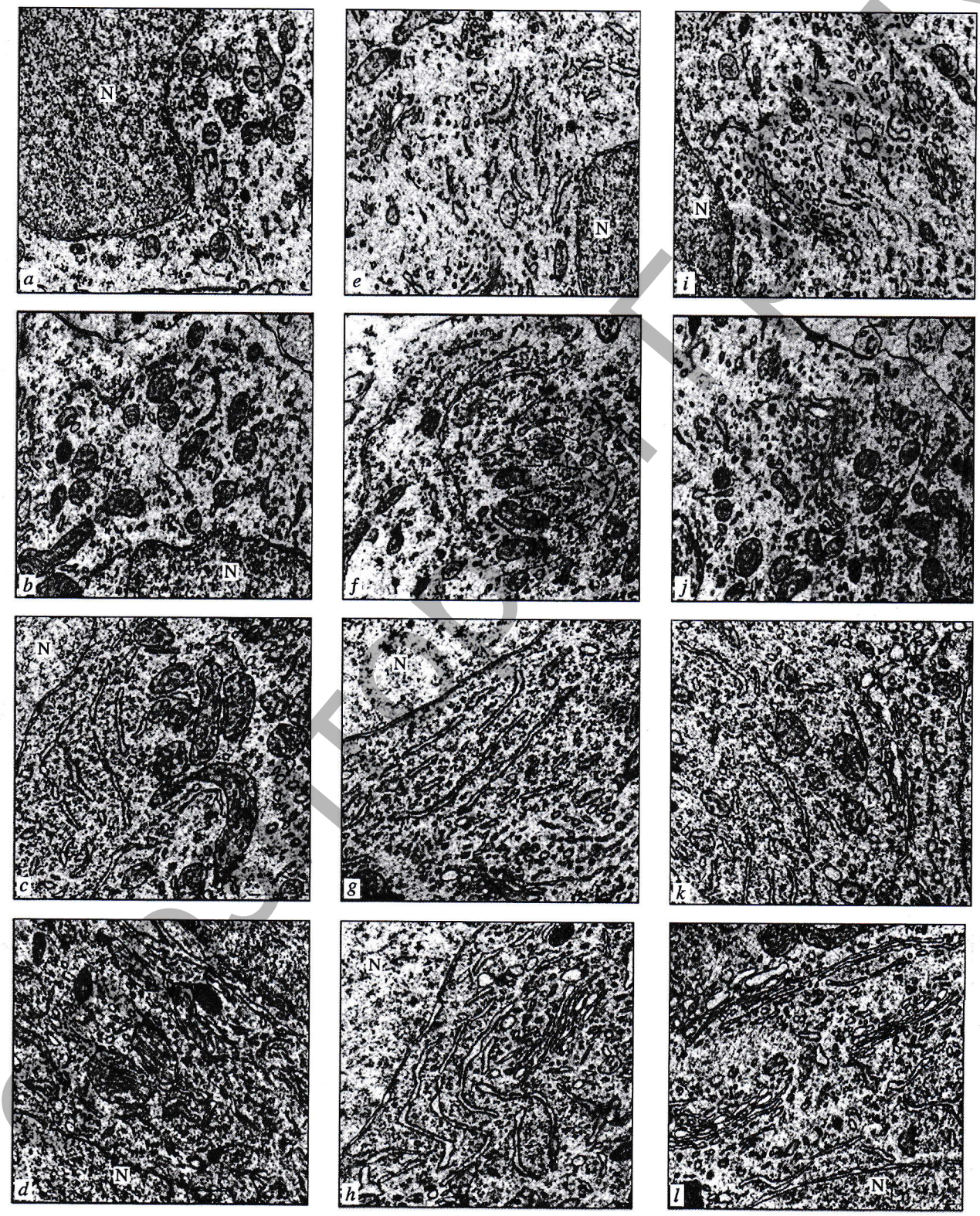

Fig. 1. Organelles in rat cerebellar Purkinje cells: *a–d*) mitochondria; *e–h*) rough endoplasmic reticulum; *i–l*) Golgi complexes. *a*, *e*, *i*) on day 2; *b*, *f*, *j*) day 7; *c*, *g*, *k*) day 15; *d*, *h*, *l*) day 45 of postnatal development; N = nucleus. Magnification: ×40000.

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TABLE 2. Postnatal Organellogenesis in Purkinje Cells (PC) in the Rat Cerebellum (Me ± IQR)

Structure of PC cytoplasm	Indicators	Age of rat, days			
		2	7	15	45
Mitochondria	Number of 1 μm² of cytoplasm	2.60 ± 1.25	1.19 ± 0.05*	1.37 ± 0.16*#	1.07 ± 0.24*+
	Mean area, μm²	0.04 ± 0.01	0.06 ± 0.04	0.07 ± 0.03*	0.09 ± 0.01*#
	Elongation factor	1.46 ± 0.55	1.55 ± 0.52	1.78 ± 0.62	2.62 ± 0.85*#
	Form factor	0.87 ± 0.10	0.83 ± 0.07	0.83 ± 0.15	0.68 ± 0.14*#
	Number of cristae per 1 μm² of mitochondria	55.21 ± 10.07	51.22 ± 19.64	42.38 ± 5.83*	56.77 ± 13.51+
	Length oi cristae per 1 μm² of mitochondria	7.50 ± 1.36	8.87 ± 1.59	8.91 ± 1.96	11.29 ± 4.69*#+
	Proportion of cytoplasmic area occupied, %	8.18 ± 4.29	6.68 ± 4.72	10.35 ± 4.91	9.75 ± 3.25#
Ribosomes	Bound, per 1 μm of RER cistern	37.06 ± 7.69	34.18 ± 4.70	31.38 ± 3.48	38.81 ± 18.13
	Free, per 1 μm² of cytoplasm	61.98 ± 21.78	70.18 ± 27.52	62.51 ± 11.34	71.77 ± 30.13
Rough endoplasmic reticulum (RER)	Cistern length	0.25 ± 0.06	0.34 ± 0.05	0.45 ± 0.04*#	0.86 ± 0.26*#+
Golgi complex	Cistern width	0.03 ± 0.00	0.04 ± 0.00*	0.05 ± 0.01*#	0.05 ± 0.01*#
Lysosomes	Number per 1 μm²	0.03 ± 0.06	0.02 ± 0.06	0.02 ± 0.03	0.10 ± 0.02*
	Mean area, 1 μm²	0.03 ± 0.01	0.06 ± 0.01*	0.04 ± 0.01*#	0.07 ± 0.03*+
	Proportion of cytoplasmic area occupied, %	0.06 ± 0.18	0.14 ± 0.32	0.09 ± 0.11	0.68 ± 0.12*#+

plasm varied by 6–10% at different times (Table 2). The round shape of these organelles was typical for the early period of postnatal ontogeny (days 2–15) and then changed to a more elongated shape (day 45) (see Table 2). The relative number of cristae per 1 μm² of mitochondria area showed almost no change from day 2 to day 45, though length increased 1.5-fold by day 45 (see Table 2). Concordantly, there was a progressive increase in the activity of the mitochondrial enzyme SDH in cerebellar PC cytoplasm during postnatal ontogeny (0.10 ± 0.02 OD units on day 2, 0.21 ± 0.03 on day 7, 0.22 ± 0.02 on day 15, and 0.21 ± 0.02 on day 45).

From day 2 to day 45, there were progressive increases in mean RER cistern length, while the number of membrane-bound ribosomes per 1 μm of RER cistern remained almost unchanged (see Table 2), though there were more free ribosomes in the cytoplasm.

On days 2 and 7, GC in cerebellar PC consisted of accumulations of vacuoles and short, narrow cisterns; on days 15 and 45 it consisted of longer, parallel cisterns, which increased in number. GC cistern width gradual increased from day 2 to day 45 (see Fig. 1, *i–l*; Table 2).

On days 2–15 of postnatal development, the number of lysosomes per unit area of cytoplasm and their specific area in the cytoplasm remained very low, while there was a sharp increase by day 45. A small increase in lysosome areas in PC was seen in seven-day-old rat pups as compared with the area in two-day-old animals; by day 15, area became smaller, and then increased almost twofold by day 45 than at day 15 (see Table 2).

Discussion. Thus, postnatal differentiation of PC was accompanied by changes in ultrastructure. As neurons matured, the areas of their perikarya increased to a greater extent than the areas of their nuclei, which led to a progressive decrease in the nucleus-cytoplasm ratio.

Immature PC were characterized by small quantities of cytoplasm in which the endoplasmic reticulum had not formed, with a significant predominance of free ribosomes over bound, and small, round mitochondria. At the initial stage of PC development, the predominance of free ribosomes was due to the biosynthesis of proteins determining the morphogenetic processes underlying cell differentiation. Prolonged persistence of large numbers of free ribosomes in PC is accompanied by a long period of growth of the dendrites of these cells [1]. The main indicator of neuron maturation is an increase in the number of membrane-bound ribosomes, and formation of the RER is linked with the biosynthesis of proteins supporting synaptic transmission. From day 2 to day 45, there was a decrease in the density of mitochondria in the cytoplasm, which led to a significant (four-fold) increase in perikaryon area. The area occupied by these organelles in the cytoplasm showed only a small change, which was associated with increases in organelle size. There was also an increase in the number and lengths of cristae, accompanied by an increase in cytoplas-

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mic SDH activity (an enzyme of the Krebs cycle), which is required for supplying energy for the functions of mature PC. Decreases in the form factor and increases in the elongation factor of mitochondria were due to change in their shape from round to extended. By day 45, there was a threefold (compared with two-day-old rats) increase in the number of lysosomes per 1 μm^2 of cytoplasm, which is evidence for the formation of intracellular digestive machinery and the systems required for removal (via autophagy) of damaged membranes and organelles. During postnatal ontogeny, the process of PC differentiation is accompanied by the formation and growth of GC and the ordered positioning and widening of its cisterns.

The quantitative results obtained here are consistent with and add to existing data on rat cerebellum PC maturation during postnatal ontogeny [4, 9]. The most informative morphometric indicators of PC maturity were: the decrease in the nucleus:cytoplasm ratio, the increase in nucleolar area, the growth in mitochondrial size and length, the increases in the number of membrane-bound ribosomes, the lengths of RER channels, the increases in the number and size of ribosomes, and the relative area occupied by lysosomes in cytoplasm.

Thus, morphometric studies of cell ultrastructure to describe the dynamics of postnatal organellogenesis in cerebellar PC provides for object assessment of the process of the postnatal differentiation of PC in the rat cerebellum, which is accompanied by increases in the complexity of the synthetic and energy-providing apparatus and the intracellular digestion and defense system. This method is effective and can also be used for studies of the effects of various experimental treatments and pathological states on the postnatal morphogenesis of the cerebellum.

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