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Synaptogenesis in the Developing Rat Cerebellum

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The aim of the present work was to perform a qualitative and quantitative evaluation of synaptogenesis in the developing rat cerebellum (2–45 days after birth) by immunohistochemical detection of the marker synaptophysin (Syn). Syn expression was detected in postmitotic neurons in the external granular layer and migrating precursors of cerebellar granular neurons. The width of the synaptogenesis zone in the molecular layer increased throughout the study period, and this was accompanied by a decrease in Syn immunoreactivity. There was also a reduction in the number of Syn-immunopositive synapses around the perikarya of Purkinje cells from day 7 to day 15. The inner granular layer showed Syn-immunopositive dots, whose sizes increased from day 2 to day 45, which was associated with the formation of cerebellar glomeruli. The nucleus interpositus of the cerebellum showed an increase in the number and size of axosomatic synapses around neuron perikarya throughout the study period. Uneven groupings of Syn-positive axodendritic synapses appeared in the neuropil.

Keywords: cerebellum, development, synaptogenesis, synaptophysin.

The development of the cerebellum during ontogeny is accompanied by the proliferation, migration, and differentiation of neurons and the formation of synaptic connections between them. Precursors of Purkinje cells (PC) form on days 13–16 of embryogenesis in rats in the ventricular layer of the roof of the fourth ventricle and then migrate in the radial direction, initially forming a layer in the future cerebellar cortex, this layer consisting of multiple rows of cells; it is only by days 3–4 of postnatal development that the layer comes to consist of a single row of cells. The formation of synapses between PC and parallel fibers (the axons of granular neurons) starts in the deep part of the molecular layer of the cerebellar cortex on days 7–9 of the postnatal period of ontogeny [9]. In rats, basket neurons form from day 2 to day 17 of postnatal development and stellate neurons from day 4 to day 19. They then differentiate and form synaptic contacts with PC [14]. Climbing fibers (axons of neurons in the interior olive) reach the cortex of the developing cerebellum and establish synaptic contact with PC by days 18–19 of embryonic development. Then, by day 15 of postnatal development, the multiple innervation by climbing fibers present at birth changes to monoinnervation [5]. Mossy afferent fibers in the internal granular layer (IGL) of the cerebellum form glomeruli. There are several sources of mossy fibers: neurons in the spinal cord (fibers of the spinocerebellar tract, which penetrate the cerebellum on day 15 of embryonic development), the vestibular neurons (vestibulocerebellar afferent fibers – on days 13–15), the nuclei of the reticular formation (reticulocerebellar projections – on days 16–17), the nucleus of the trigeminal nerve (on day 22 of intrauterine development), and the nuclei of the pons (pontocerebellar afferent fibers – after birth) [12].

Two stages are distinguished during differentiation of cerebellar glomeruli: the protoglomerular and the glomerular. The first stage occurs in rats during the first two weeks of postnatal development and is characterized by rapid expansion of mossy fiber rosettes, which contain synaptic vesicles close to the plasmalemma. The second stage (the glomerular) involves the formation and stabilization of glomeruli – from day 15 to day 45. During this time, there are increases in the number of synaptic vesicles in mossy fiber terminals and in contacts with the dendrites of granular cells, which

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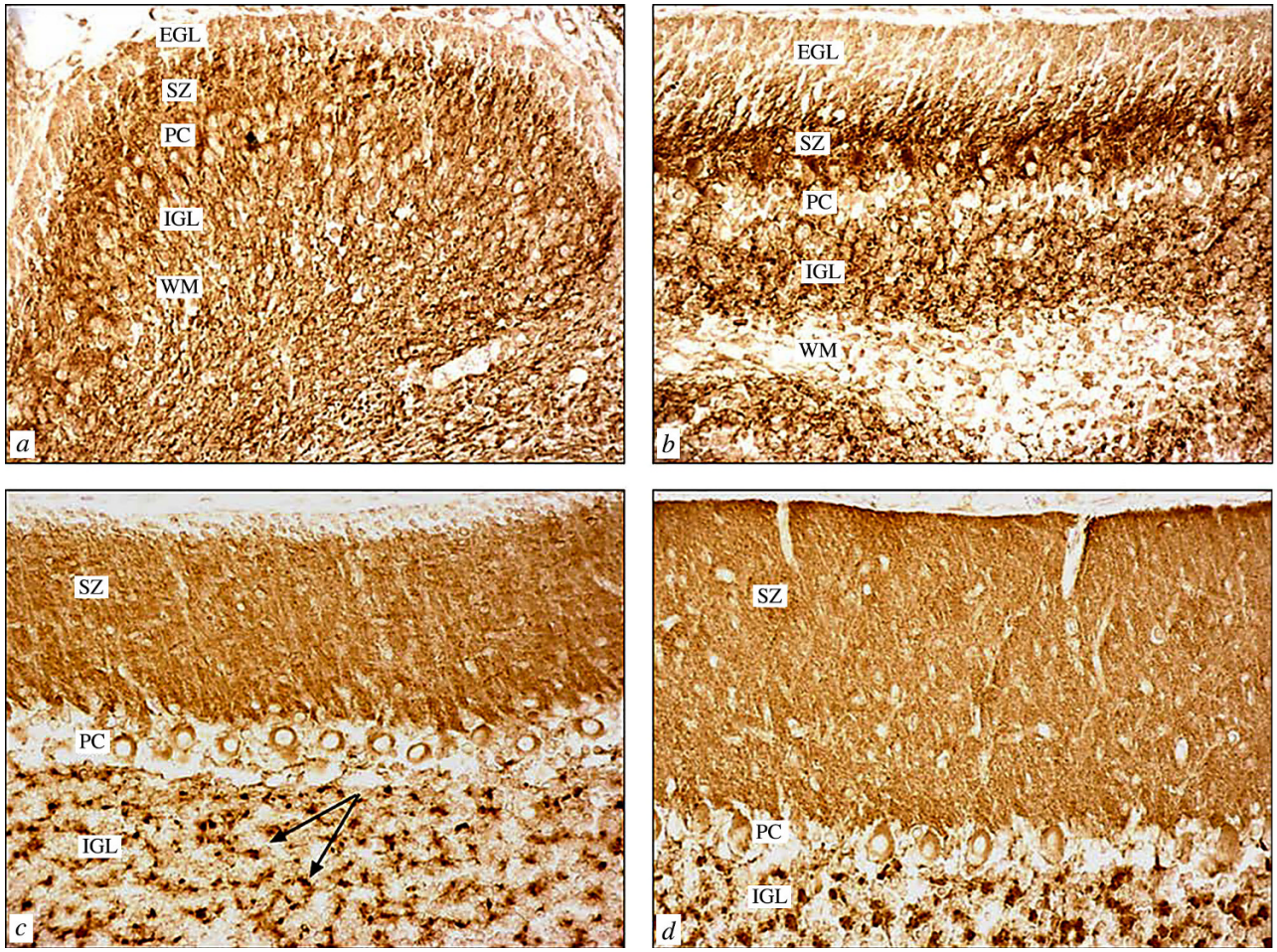

Fig. 1. Expression of synaptophysin (Syn) in the paleocerebellum in rats on days 2 (*a*), 7 (*b*), 15 (*c*), and 45 (*d*) after birth. EGL – external granular layer; SZ – synaptogenesis zone in the molecular layer; PC – Purkinje cells; IGL – internal granular layer; WM – white matter; arrows show cerebellar glomeruli. Immunohistochemical reaction for Syn. Magnification ×200.

form blebs (spines) containing mitochondria and endoplasmic reticulum cisterns [2]. The efferent pathways of the cerebellum (formed by PC axons) start to form in the late period of embryogenesis, and PC establish synaptic contacts with target cells (neurons of the cerebellar nuclei) by day 20 of intrauterine development [7].

The molecular marker for synaptic vesicles, synaptophysin (Syn) allows synaptogenesis to be characterized during the development of the cerebellum [1, 3, 11]. Syn accounts for 10% of all protein in synaptic vesicles and supports their contact with the presynaptic membrane; it is involved in neurotransmitter endo- and exocytosis [10]. Embryonic Syn differs from Syn of adult animals in terms of its biological properties [6]. The presence of Syn is not obligatory for the formation of synaptic vesicles [8]. The current view is that the absence of Syn produces no changes in transmitter exocytosis, though endocytosis is impaired after transmission of a nerve spike and refilling of synaptic vesicles with transmitter is slowed, which limits the capacity for nerve spike transmission by neurons [10]. Syn is synthesized in perikarya and then enters the axons of neurons [9]. Expression of Syn in the molecular layer of the cerebellum is due mainly to synaptic contacts between the dendrites of PC and parallel fibers, while that in the granular layer is due to mossy fibers [11].

The aim of the present work was to assess synaptogenesis in the developing rat cerebellum by immunohistochemical detection of Syn.

Materials and Methods

Experiments were performed on the offspring of female mongrel white mice with initial weights of 180 ± 20 g. All experiments were performed in compliance with the "Regulations for Studies Using Experimental Animals." This study was approved by the Biomedical Ethics Committee of the 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 taken from each female on reaching 2, 7, 15, and 45 days after birth and was decap-

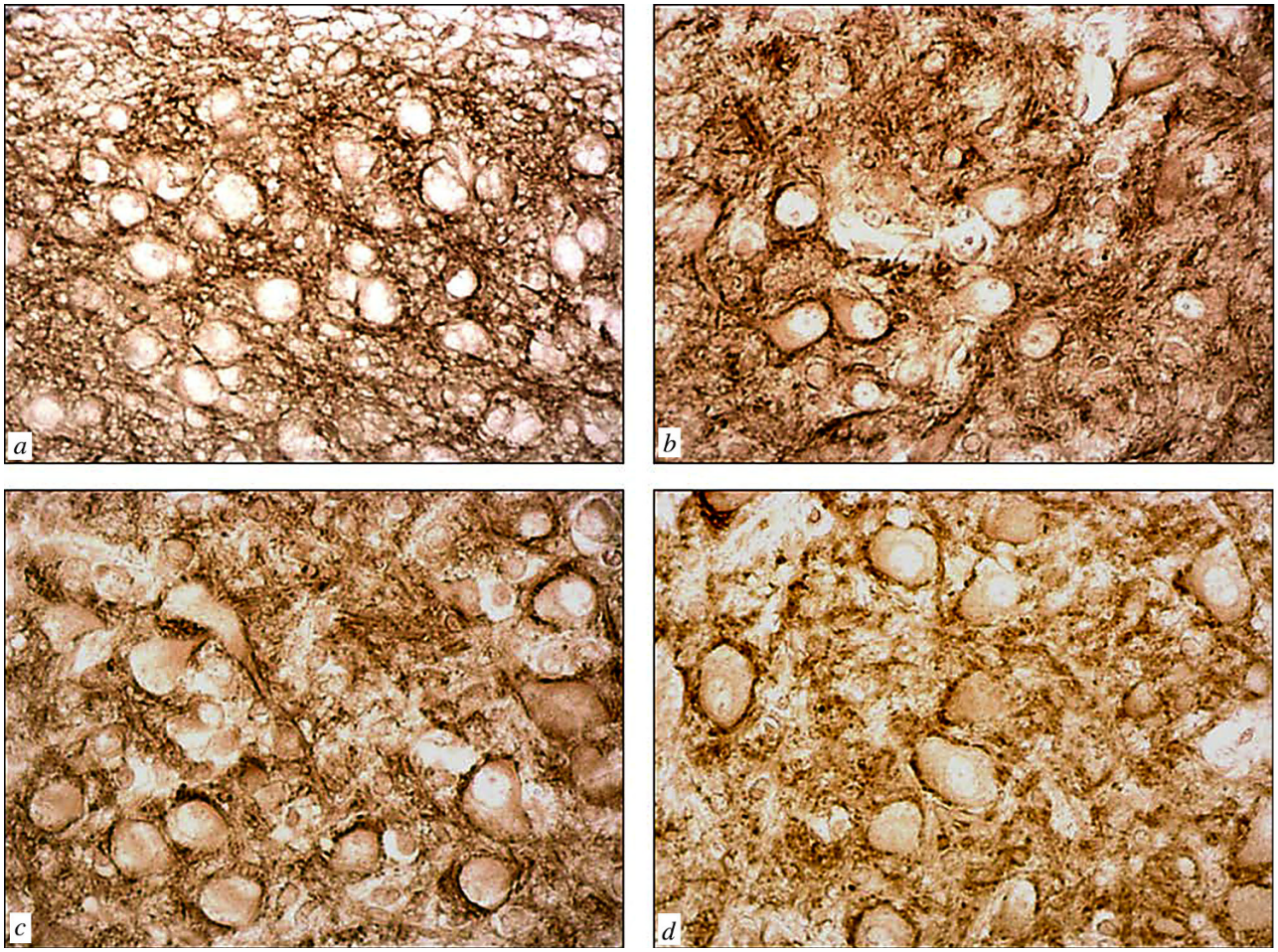

Fig. 2. Synapses on the bodies and dendrites of neurons in the nucleus interpositus of the cerebellum on days 2 (*a*), 7 (*b*), 15 (*c*), and 45 (*d*) after birth. Immunohistochemical reaction for synaptophysin. Magnification ×400.

TABLE 1. Characteristics of the Synaptogenesis Zone in the Molecular Layer of the Cortex in the Paleo- and Neocerebellum in Rats during Postnatal Ontogeny (Me ± IQR)

Parameter	Age of pups, days	Paleocerebellum	Neocerebellum
Zone width, μm	7	29.22 ± 8.45	24.19 ± 5.64
	15	81.99 ± 12.03*	78.03 ± 13.01*
	45	170.47 ± 17.83*	146.56 ± 20.48*
Immunoreactivity, OD units	7	0.29 ± 0.02	0.32 ± 0.04
	15	0.25 ± 0.05*	0.23 ± 0.02*
	45	0.22 ± 0.03*	0.23 ± 0.02*

*Significant differences compared with values in seven-day-old rat pups.

itated (a total of 12 pups were studied). Comparable results from each animal were obtained by processing fragments of cerebellum in parallel in identical conditions. These were fixed in zinc-formalin at 4°C (overnight) and then embedded in paraffin. Sections of thickness 7 μm were cut with a Leica RM 2125 RTS microtome (Leica, Germany). Immunohistochemical detection of Syn was with primary rabbit polyclonal antibodies, Synaptophysin Antibody (PA5-

TABLE 2. Numbers of Cerebellar Glomeruli in the Internal Granular Layer of the Cerebellum (Me ± IQR per mm^2 of sections)

Age of pups, days	Cerebellar glomeruli	Paleocerebellum	Neocerebellum
15	Large	121.84 ± 97.97	124.65 ± 77.29
	Intermediate	2193.16 ± 1411.22	2476.98 ± 2278.41
	Smal	785.60 ± 1137.94	1227.77 ± 1000.18
	Total	3568.80 ± 733.23	3757.72 ± 896.52
45	Large	472.38 ± 11.32*	434.39 ± 30.84*
	Intermediate	2909.60 ± 305.09	3185.52 ± 893.87
	Smal	356.66 ± 88.31*	732.23 ± 226.61*
	Total	3715.36 ± 206.42	4394.87 ± 515.69

*Significant differences compared with values in 15-day-old rat pups, $p < 0.05$.

27286) (Thermo Scientific, USA), diluted 1:400 at 4°C, exposure for 20 h, in a moist chamber. Bound primary antibody was detected using a Thermo Scientific Super Picture™ Polymer Detection Kit. The locations of the phylogenetically ancient (paleocerebellum) and new (neocerebellum) parts of the cerebellum on histological sections of the developing cerebellum in rat pups were determined on the basis of data reported by Olenev [4] using the Paxinos and Watson atlas [13]. Histological sections were examined, microphotographed, and studied morphometrically using an Axioscop 2 plus microscope (Zeiss Germany) with a LeicaDFC 320 digital video camera (Leica, Germany) and image analysis program ImageWarp (Bitflow, USA). The density of cerebellar glomeruli per mm^3 of the IGL was determined in rat pups, along with the width of the synaptogenesis zone and levels of Syn expression in the molecular layer of the rat cerebellum, the number of synapses around PC perikarya, and Syn expression in the neuropil of the nucleus interpositus. Mean values obtained in animals of each experimental group were analyzed by nonparametric statistics in Statistica 6.0 for Windows (StatSoft, USA). The descriptive statistics for each parameter included determination of the median (Me) and interquartile range (IQR).

Results

On days 2 and 15 after birth, the outer rows of the external granular layer (EGL) in the rat cerebellar cortex, formed by dividing cells – granular neuron precursors – showed no staining in the reaction for Syn. Syn was detected in the cytoplasm of future granular cells ready for migration or already migrating through the molecular layer (Fig. 1, *a–c*). Syn immunoreactivity was also present in the cytoplasm of granular cells in the IGL, though the level decreased with increasing age (days 15–45). (see Fig. 1).

On day 7, Syn staining of both the paleo- and neocerebellum in control rats revealed synapses as densely distributed dots between PC perikarya and in the deep part of the molecular layer (Fig. 2, *b*). On day 15 and especially day 45 of postnatal ontogeny, thickening of the molecular layer in both parts of the cerebellum was accompanied by an increase in the width of the synaptogenesis zone (Table 1). Syn-immunopositive synapses became smaller, more numerous, and were distributed more uniformly on immunonegative PC dendrites as compared with day 7, while stained synaptogenesis zones became more homogeneous and weaker (see Fig. 1, Table 1), except in bands of increased immunoreactivity on the surface of the molecular layer on day 45 (see Fig. 1, *d*).

On days 2 and 7 after birth, Syn expression was observed between PC perikarya (see Fig. 1, *a*). On day 7, Syn-immunopositive synapses in the form of numerous large, intensely stained dots, were visible around the perikarya of Purkinje cells – forming axosomatic synapses. On days 15–45 after birth, the numbers of axosomatic synapses on PC perikarya decreased significantly. At these times, the numbers of large Syn-positive synapses on PC perikarya in the paleo- and neocerebellum amounted to 2.0 ± 1.0 (Me ± IQR). At this time, small Syn-immunopositive axosomatic synapses dominated, apparent as fine powder-like granularity. Syn immunoreactivity was also detected in the cytoplasm of PC: on day 2 it was weak, then increased sharply, reaching a maximum on day 7, and then gradually decreased. The perikarya of PC were clearly seen on the background of a light neuropil with weak immunoreactivity (see Fig. 1, *c*, *d*).

Syn immunoreactivity in the forming IGL was weak on day 2 of postnatal ontogeny in rats and was apparent as fine granularity, no different from that in the white matter. On day 7, mainly small Syn-immunopositive dots were distributed among granular neurons – evidently mossy fiber terminals, which are the basis for the formation of future cerebellar glomeruli (see Fig. 2, *b*). On day 15, Syn-immunopositive fine and intermediate-sized forming cerebellar glomeruli were identified among granular cells (see Fig. 2, *c*).

TABLE 3. Expression of Synaptophysin (Syn) in the Neuropil of the Nucleus Interpositus of the Cerebellum in Rats during Postnatal Ontogeny (Me ± IQR, optical density units)

Age of pups, days	Syn expression
2	0.22 ± 0.03
7	0.23 ± 0.03
15	0.22 ± 0.01
45	0.21 ± 0.04

By day 45, the number of large Syn-positive glomeruli with very high immunoreactivity increased, while the number of small glomeruli decreased (see Fig. 1, *c*; Table 2). There was no change in the total number (density) of glomeruli in the IGL (see Table 2).

In the nucleus interpositus of the cerebellum, neuron perikarya on day 2 after birth showed only occasional Syn-immunopositive dots, i.e., axosomatic synapses. Neurons with small numbers of axosomatic synapses dominated, while there were only occasional neurons with densely distributed synapses. In the neuropil there was a uniform distribution of numerous small axodendritic synapses. On days 7–45, dark rims formed around the perikarya of some neurons, these consisting of densely distributed axosomatic synapses (see Fig. 2). From day 7 to day 14, the size of axodendritic synapses in the neuropil increased and their distribution started to become non-uniform, forming groups (see Fig. 2). Overall neuropil immunoreactivity in the nucleus interpositus of the cerebellum changed during postnatal ontogeny (Table 3). Syn immunoreactivity in the cytoplasm of neurons in this structure was moderate, homogeneous, and unaltered throughout the observation period (see Fig. 2).

In the white matter of the cerebellum, Syn immunoreactivity was detected on day 2 and, to a lesser extent, on day 7 of postnatal development, which was particularly clearly apparent in Crus I on day 7 after birth. This subsequently disappeared.

Discussion

The absence of Syn immunoreactivity in the outer rows of the EGL on days 2–15 after birth is evidence that this glycoprotein is expressed in the cytoplasm and that nerve terminals are not present in the zone in which granular neuron precursors proliferate. However, immunoreactivity was seen in the cytoplasm of premigratory and migrating granular neuron precursors. This is supported by the fact that Syn in EGL neurons, located in the premigratory zone, was seen in the translateral cisterns of the Golgi complex and close to it in synapse-like vesicles and persisted in these same neurons migrating through the molecular layer [9].

In our study, the immunohistochemical reaction for Syn produced staining around the perikarya of PC and in the forming molecular layer on day 2 of postnatal ontogeny. The literature contains descriptions of electron microscope studies showing that the first synapses in the molecular layer are detected by day 19 of intrauterine development [15]. It is possible that mossy fiber terminals on PC enter the cerebellum before birth. Contacts with them are temporary: when the granular layer matures, mossy fibers are displaced from PC and establish synapses with the dendrites of granular neurons [12].

The increase in the width of the synaptogenesis zone and the decrease in Syn-immunoreactivity within it from day 7 to day 45 after birth is evidently linked with the growth of the dendritic trees of PC. The decrease in the number of Syn-immunopositive synapses around PC perikarya from day 7 to day 15 may be due to elimination of excess innervation by climbing fibers and the formation of synapses on PC dendrites by these fibers.

In the IGL, microgranular Syn immunoreactivity on days 2–7 of postnatal ontogeny is probably due to growth cones and mossy fiber terminals, while on days 15–45 it is probably due to forming cerebellar glomeruli [2], which are apparent as larger areas of high Syn immunoreactivity.

The increase in the number of Syn-positive synapses on the perikarya of neurons in the nucleus interpositus of the cerebellum during postnatal ontogeny is evidently associated with the formation of efferent fiber terminals running from PC, occurring at times consistent with those reported in the literature [7]. In addition, mossy fiber and climbing fiber collaterals terminate on neurons in the cerebellar nuclei, as do multilamellar (neuromodulatory) fibers. The latter include serotoninergic (formed postnatally), noradrenergic (enter the cerebellum on day 17 of intrauterine development), and cholinergic, dopaminergic, and histaminergic [12] fibers. Our data indicate that total Syn expression in the neuropil of the nucleus interpositus of the cerebellum in rats does not change during postnatal ontogeny, which is linked with enlargement of synapses and decreases in their number (distribution density).

The Syn immunoreactivity in the white matter on days 2–7 after birth is probably due to the growth cones of developing afferent fibers. Synaptic vesicles are known to form in cell bodies and then to be transported along growing axons to accumulate in growth cones before the onset of syn-

aptogenesis [9]. This explains the presence of moderate Syn immunoreactivity in the cytoplasm of the perikarya but not the nuclei of cerebellar neurons (PC, granular neurons, neuron nuclei).

Thus, immunohistochemical analysis of the dynamics of Syn expression is an effective means of evaluating synaptogenesis in the cerebellum during the early postnatal period of ontogeny. This approach can also be used in studies of the effects of various experimental interventions and pathological states on this process.

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