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Expression of Doublecortin and NeuN in Developing Neurons in the Rat Cerebellum

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Experiments were performed on the offspring of five mongrel white rats using comparative immunochemical assessments of doublecortin (DCX) and neuronal nuclear antigen (NeuN) expression in neurons in the cortex and nucleus interpositus of the cerebellum in animals during early postnatal ontogeny (days 2–15). DCX expression was seen in postmitotic neurons in the external granular layer and migrating neurons in the cerebellar cortex. DCX expression was greater in the neocerebellum than in the paleocerebellum in rat pups aged two and seven days. NeuN expression was seen in migrating granule neurons, reaching a maximum in more mature neurons in the internal granular layer. DCX expression was not seen in Purkinje cells or in neurons in the nucleus interpositus of the cerebellum. Neurons in the nucleus interpositus showed progressive increases in NeuN from day 2 to day 15 after birth. Thus, comparative immunohistochemical studies of the dynamics of the expression of this pair of molecular markers provide an effective means of evaluating the development of cerebellar granule neurons during early postnatal ontogeny.

Keywords: cerebellum, neurons, development, doublecortin, NeuN.

Molecular markers are ever more widely used to assess brain development during ontogeny in animals and humans [2–4]. Doublecortin (DCX) is a protein associated with cytoskeletal microtubules in vertebrate neurons and expressed by immature neurons before and during migration during brain development. It was subsequently detected in growing neuron processes [7]. DCX is also expressed in brush neurons in the vestibulocerebellum in adult rats and plays a particular role in the plasticity of these cells [13]. NeuN (neuronal nuclear antigen) is a neuronal nuclear antigen which is expressed in the nuclei and perinuclear cytoplasm of most granule cells in the CNS but is not detected in immature nerve cells. NeuN is not expressed in Purkinje cells or neurons in the dentate nucleus of the cerebellum, or in glial cells [12].

The cortex of the developing cerebellum in rats contains, along with the usual, "internal" granular layer (the IGL), an additional external granular layer (the EGL), where cell proliferation persists into the postnatal period. The EGL of the cerebellum forms in 17-day-old embryos as a result of the first wave of migration to the outer surface of the developing cerebellum from the dorsal part of the neural tube, i.e., the rhombic lip, located on the boundary of the mesencephalon and telencephalon [16]. The EGL is a germinative zone consisting of 3–6 rows of granule neuron precursors [10]. The 1–3 outer layers form proliferating cells which, after their last mitosis, are discharged into the inner rows of the EGL, acquire a fusiform shape, and initiate radial migration to the future IGL [11]. The EGL reaches its maximal development by day 7, and most of the granule neurons making up the IGL form between days 7 and 15 of postnatal development; the EGL in rats disappears by postnatal days 20–21. A number of studies have established that the migratory behavior of granule cells is due to a genetic program [17], and is also influenced by the microenvironment, activation of specific N-methyl-D-aspartic acid (NMDA) receptors, and the polymerization and degradation of microtubules creating pulling and pushing forces on the nucleus and cytoplasm [14].

The aim of the present work was to carry out a comparative evaluation of the dynamics of DCX and NeuN expression in the cortex of the paleocerebellum and neocerebellum

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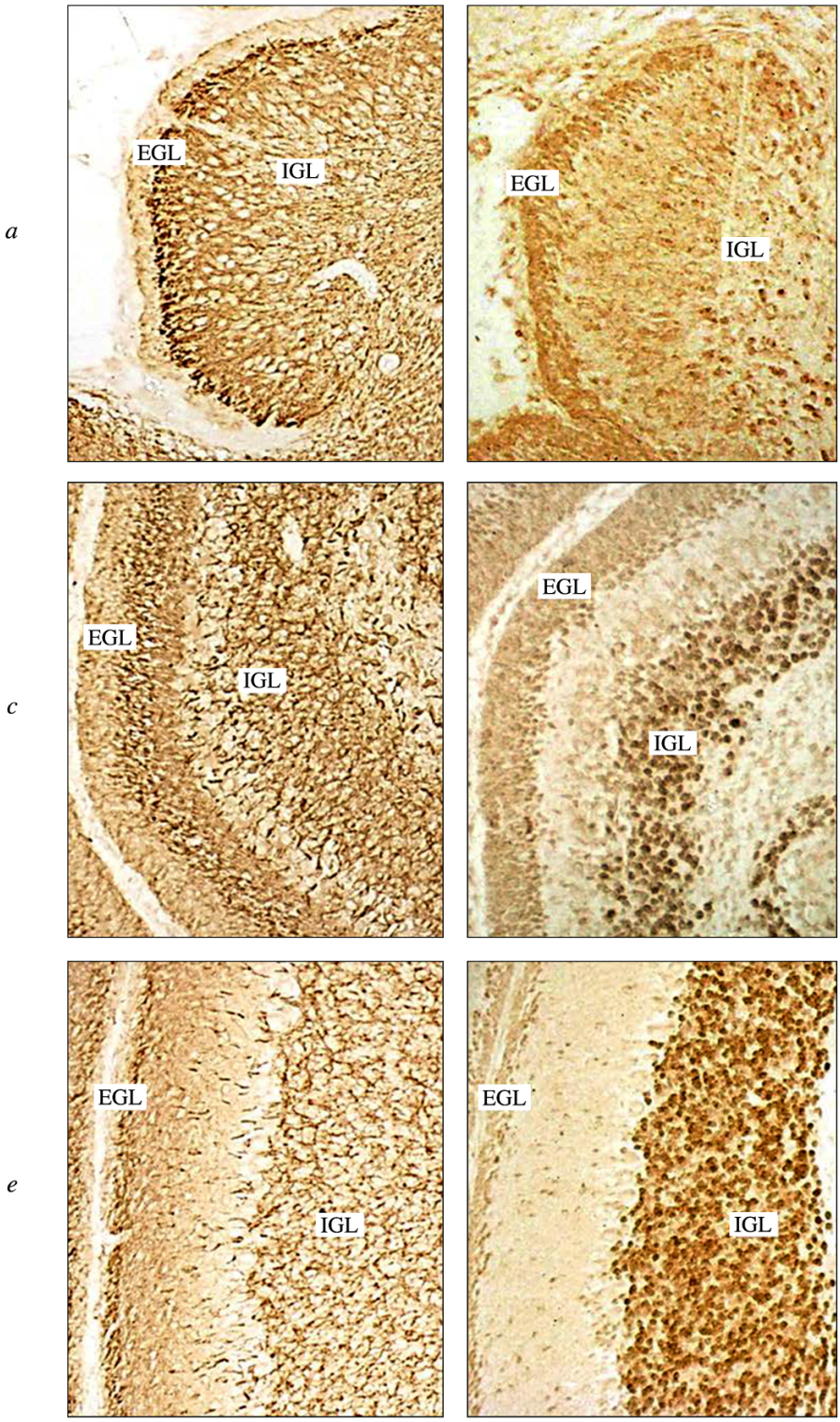

Fig. 1. Expression of doublecortin (DCX) and neuronal nuclear antigen (NeuN) in the cortex of the paleocerebellum in rat pups aged two (*a*, *b*), seven (*c*, *d*), and 15 days (*e*, *f*). EGL – external granular layer; IGL – internal granular layer. Immunocytochemical reactions: *a*, *c*, *e*) for DCX; *b*, *d*, *f*) for NeuN. Magnification ×100.

TABLE 1. Expression of Doublecortin in Premigratory Neurons in the External Granular Layer of the Cerebellar Cortex in Early Postnatal Ontogeny in Rats (Me ± IQR, OD units)

Time after birth, days	Part of cerebellum	
	Paleocerebellum	Neocerebellum
2	0.25 ± 0.03	0.33 ± 0.05^{+}
7	0.20 ± 0.020*	0.24 ± 0.04*$^{+}$
15	0.190 ± 0.010*	0.190 ± 0.020*$^{\#}$

Here and in Tables 2–4: significant differences compared with *two-day-old rats; $^{\#}$seven-day-old rats; $^{+}$the paleocerebellum, $p < 0.05$.

TABLE 2. Characteristics of Cerebellar Neurons in Rats during Postnatal Ontogeny (Me ± IQR)

Parameter	Period after birth, days	Part of cerebellum		
		Paleocerebellum	Neocerebellum	Nucleus interpositus
Number of NeuN-positive neurons in the IGL of the cortex per 1000 μm^2 section area	2	4.4 ± 1.8	3.9 ± 0.5	–
	7	8.89 ± 0.28*	9 ± 4*	–
	15	14.1 ± 1.6*$^{\#}$	15.4 ± 2.1*$^{\#}$	–
NeuN expression in neurons, OD units:	2	0.14 ± 0.06	0.10 ± 0.10	0.130 ± 0.010
in the IGL of the cortex	7	0.20 ± 0.09	0.18 ± 0.03	0.140 ± 0.010
in the nucleus interpositus	15	0.25 ± 0.04*$^{\#}$	0.26 ± 0.04*$^{\#}$	0.170 ± 0.020*$^{\#}$

IGL – internal granular layer of the cortex; NeuN – neuronal nuclear antigen.

TABLE 3. Thickness of the Granular Layers of the Cerebellar Cortex in Postnatal Ontogeny in Rats (Me ± IQR, μm)

Granular layers	Time after birth, days	Part of cerebellum	
		Paleocerebellum	Neocerebellum
External	2	14 ± 3	13.8 ± 2.8
	7	41 ± 11*	45 ± 6*
	15	20.2 ± 1.2*$^{\#}$	26 ± 7*$^{\#+}$
Internal	2	72 ± 7	60 ± 5^{+}
	7	62 ± 8*	48 ± 6*$^{+}$
	15	125 ± 23*$^{\#}$	107 ± 24*$^{\#}$

and the nucleus interpositus of the cerebellum in rats in early postnatal ontogeny.

Materials and Methods

Experiments were performed on five female mongrel white rats with starting weights of 180 ± 20 g and their offspring. All experiments were performed in compliance with the "Regulations for Studies Using Experimental Animals" [1]. The study was approved by the Biomedical Ethics Committee of Grodno State Medical University (protocol No. 7 of December 12, 2013). Animals were kept on a standard animal-house diet. A single pup was taken from each animal at 2, 7, and 15 days after birth; pups were decapitated. To ensure comparable results, samples of cerebellum from all animals were processed together in identical conditions. Samples were fixed in zinc-ethanol-formaldehyde at 4°C (overnight) and then embedded in paraffin by standard methods. Sections of thickness 5 μm were cut with a LeicaRM 2125 RTS microtome (Leica, Germany). Immunochemical detection of DCX was with primary rabbit polyclonal antibodies (Abcam, UK) ab.18723, and NeuN was detected with antibody ab.128886 (antibodies diluted 1:400, 4°C, 20 h, moist chamber). Binding of primary antibodies was detect-

ed using an Expose Rabbit-Specific HRP/DAB detection IHC kit (ab.80437; Abcam, UK). Neighboring sections were stained with 0.1% thionine solution by the Nissl method. The thicknesses of the EGL and IGL in the cortex were measured in the paleo- and neocerebellum. Their locations on histological preparations of the developing cerebellum were identified in terms of Olenev's descriptions [5]. The optical densities of chromogen precipitates in DCX-immunopositive neurons in the EGL and NeuN-immunopositive neurons in the IGL and nucleus interpositus of the cerebellum were determined. The density of NeuN-immunopositive neurons per 1000 μm^2 was determined in the IGL.

Examination of histological preparations, microphotography, and morphometry were performed using an Axioscop 2 plus microscope (Zeiss, Germany), a Leica DFC 320 digital video camera (Leica, Germany), and the image analysis program ImageWarp (Bitflow, USA). Median and interquartile ranges (Me ± IQR) were determined for each metric. Differences between values under comparison were evaluated using the Mann–Whitney test and were regarded as significant at $p < 0.05$.

Results

On postnatal day 2, the outer 2–3 rows of the EGL of the cerebellar cortex, which contains dividing cells, lacked DCX-immunopositive neurons (Fig. 1, *a*). Intense DCX expression was seen in the 2–3 inner rows of EGL cells, which contain postmitotic granule neurons ready for radial migration (see Fig. 1, *a*; Table 1). NeuN expression was absent from these cells, though it was detected in migrating granule neurons, reaching a maximum in more mature neurons in the IGL (see Fig. 1, *b*).

On day 7 of postnatal ontogeny, when the EGL reached its maximal thickness (Table 2), its migration-ready postmitotic neurons showed decreased immunoreactivity (see Table 1). DCX expression increased in migrating neurons and the growing processes of IGL neurons (see Fig. 1, *c*). NeuN expression in the IGL was low, but increased in migrating neurons to reach a maximum in more mature neurons completing migration to the IGL (see Fig. 1, *d*).

On day 15 after birth, the thickness of the EGL decreased to half that on day 7 (see Table 2). At this time, DCX expression in migration-ready neurons decreased further. Expression was detected in migrating granule cells, but was absent from the bodies of IGL neurons. However, the intensity of the DCX reaction in the neuropil (probably growing granule neuron processes) was quite high (see Fig. 1, *e*). Moderate NeuN expression was detected in migrating granule neurons at this time, and was maximal in the bodies of IGL neurons. NeuN immunoreactivity was not detected in the neuropil of this layer (see Fig. 1, *f*).

Thus, DCX expression in premigratory neurons in the EGL of the cortex of the paleo- and neocerebellum gradually decreased from day 2 to day 15 of postnatal ontogeny. DCX expression in the neocerebellum of rat pups aged two and seven days was greater than that in the paleocerebellum, though by day 15 it was no different from that in the paleocerebellum (see Table 1).

From day 2 to day 15 of postnatal ontogeny, the density of NeuN-immunopositive neurons in the IGL increased progressively (by factors of 3–4); these cells also showed increases (by factors of 2–2.5) in NeuN expression (see Table 2).

Comparison of the cortex in the paleo- and neocerebellum identified a degree of delay in the increase in NeuN expression in the neocerebellum as compared with the paleocerebellum (see Table 2). On day 15 after birth, the EGL in the neocerebellum was significantly thicker, while the IGL was somewhat thinner than that in the paleocerebellum (Table 3).

Purkinje cells (PC) in two-day-old rat pups were positioned in several rows; by day 7, they settled to form a monolayer. Expression of DCX and NeuN proteins was not detected on day 2 after birth.

DCX expression was not seen in neurons in the nucleus interpositus of the cerebellum on days 1, 7, or 15 of postnatal development. This is evidence that by day 2 of postnatal ontogeny, the migration of these neurons was already complete and the cells were undergoing differentiation, which was accompanied by a progressive increase in NeuN expression within them (see Table 2).

Discussion

Simultaneous comparative immunohistochemical studies of the pair of molecular markers DCX and NeuN may provide a convenient approach to assessing the developmental dynamics of cerebellar neurons in rats during early postnatal ontogeny. DCX has been shown to be absent from mitotically dividing cells in the EGL; detection starts in migration-ready postmitotic neurons, with no detection in the bodies of neurons which have completed migration and are differentiating. This is consistent with published data on the appearance of DCX in migration-ready neurons and its absence in the bodies of mature neurons [15]. DCX is involved in stabilizing the microtubules required for neuron migration [8] and also for the formation of new processes; it may also be important for synaptogenesis in IGL neurons. Increases in DCX in the neuropil of the granular layer on days 7–15 coincided with the period of intense growth of neuron processes and synaptogenesis in the cerebellar cortex [9]. Conversely, NeuN expression in immature postmitotic EGL neurons is very low, but progressively increases in migrating and especially mature IGL neurons, which is consistent with published data [12].

Changes occurring in PC and, particularly, the fact that they formed a monolayer by day 7 are probably due to mechanical factors: the pressure of the thickening granular layer beneath and the barrier formed by parallel fibers above, preventing penetration of PC perikarya into the molecular layer. This process is not accompanied by translocation of the nucleus and rearrangements of the cytoskeleton [6]. The fact that expression of DCX protein was not detected in PC even on day 2 after birth is evidence that migration of PC is

complete by this time. However, NeuN expression is also not seen in early postnatal ontogeny in cerebellar PC, which is consistent with published data [12].

As maturing granule cells in two-day-old rat pups were distributed diffusely, IGL thickness was even rather greater than that in seven-day-old pups. A delay in the decrease in thickness of the EGL was noted in the neocerebellum, with decreased DCX expression in these cells on the one hand and the absence of thickening of the IGL and increased NeuN expression in its neurons on the other, as compared with the paleocerebellum. All these points are consistent with the view that the development of the paleocerebellar cortex in early ontogeny occurs earlier.

Thus, comparative immunohistochemical studies of changes in the expression of a pair of molecular markers – DCX and NeuN – is an effective method of assessing the development of cerebellar granule neurons in early postnatal ontogeny. This approach can also be used in studies of the effects of various experimental interventions on the postnatal morphogenesis of the cerebellum, as well as pathological states.

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