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The Proliferative Pattern of the Ventricular Neuro-epithelium of Embryonic Rat Fourth Ventricle

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Abstract:

Background Studies have shown that the ventricular neuroepithelium (VN) of the dorsal metencephalonrepresents the site of the cerebellar GABAergic neurons progenitors' production. These studies used different histological techniques but all have provided qualitative information regarding the biosynthesis and cell mitosis.

Objective Quantifying the proliferative activity of the cells at the (VN) during the embryonic period.

Methods Nine age groups from day 13 to day 21 albino rat embryos *Rattusrattusnorvegicusalbinus* were investigated with Ag-NOR staining technique.

Results There was a statistically significant difference ($p<0.01$) between cellular activity at different age groups with a maximum proliferative activity on the embryonic day 15.

Conclusion Ag-NOR staining technique provided a valuable quantitative index of cell proliferation at the (VN) of the developing cerebellum.

Key words Ventricular Neuroepithelium, Rhombencephalon, Ag-NOR, Quantitative

Introduction

The term rhombencephalon is widely used to designate the region of the neuroaxis that surrounds the fourth ventricle and its recesses. The developing rhombencephalon is subdivided into two parts, the myelencephalon, giving rise to the medulla, and the metencephalon, which is said to give rise to the cerebellum and the pons. The dorsal surface plates of the rhombrncephalon fail to fuse medially. Instead, a membrane, the medullary velum, part of which becomes the telachoroidea, spreads over the enlarged rhomboid cavity, the fourth ventricle. This membrane interconnects the edges of the caudal and the rostral portions of the classical dorsal rhombencephalon. The bridgeheads are the dorsal metencephalonrostrally and the precerebellarneuroepithelium caudally [(1)].

Different morphological descriptions and delineations have been given to the roof plate of the developing rhombencephalon, the dorsal metencephalic anlage [(2,3)] and the cerebellar analage[(4)].

The dorsal metencephalonisis the site of development of the cerebellar and extracerebellar structures. This is in agreement with the current findings of embryogenesis in the roof plate of the metencephalon[(5)].

The dorsal metencephalon is lined by the ventricular neuroepithelium (VN) that begins its productive activity on the embryonic day 13 that is considered to be the onset of the formation of the cerebellar primordium in the rat[(6)]. Both morphological and genetic approaches suggest that VN is the source of all GABAergic types of cells in the developing cerebellum [(7,8)].These GABAergic neurons

consist of at least five different neuronal subtypes and are generated in three sequential but overlapping waves, the first-born are small Deep Cerebellar Nuclei neurons which eventually settle in the white matter beneath internal granule neurons. Purkinje cell progenitors are second to be generated and they become postmitotic then migrate dorsally along guiding radial glial processes to their final destination beneath the external granule layer. A third population of neurons, which consists of GABAergic interneurons of the Deep Cerebella Nuclei, stellate, basket, Lugaro, and Golgi cells, is generated during late embryonic and postnatal development[9].

In the proliferating cells, nucleolar organizer regions (NORs) are loops of DNA which contain ribosomal RNA genes important for the synthesis of proteins. These NORs are stained with silver colloid technique, and the result is known as Ag-NOR dots [10].AgNOR (Argyrophilic nuclear organizers regions) technique is simple, rapid, inexpensive and can be performed on paraffin- embedded tissue including archival material. Therefore, unlike most available 'proliferation' techniques, it does not require special processing of tissue [11]. By using this technique these NOR-associated proteins are selectively stained; and the number and area of Ag-NORs are an accurate index of activity and cell proliferation in terms of protein synthesis [12,13]. Hence, Ag-NOR stain is used to measure the biosynthetic profile and cell mitotic activity by demonstrating the amount of rRNA that increases during cell replication[14]. It can be used as marker for both cell proliferation and malignancy [15].The Ag-NOR staining technique was employed as a quantitative method as it is recommended for the investigation of protein biosynthesis and cellular activity in the developing CNS [16].

This work aims at assigning a quantitative proliferation index for the cells of the VN during their embryonic development by the application of Ag-NOR staining technique and correlate it to other qualitative studies.

Methods

A sample of eighteenth albino rats *Rattusrattusnorvegicus*albinus was divided into nine age groups from embryonic day 13 to embryonic day 21 and brain tissue specimens were obtained by decapitation. Tissue blocks were immersed in Bouin's fixative for 16 hours at room temperature (25°C) and parasagittal paraffin sections of 6 micrometer thickness were prepared for embryonic age day 13 through day 21.Sections were stained according to the method of Ploton[17]. Dewaxingin xylene was done for 3-5 minutes then pre-incubation in glycine solution (made by dissolving glycine powder (AnalaR) in 99% ethanol alcohol) for 10-20 minutes followed by rehydration in descending concentrations of ethanol alcohol (100%, 90%, 80% and 70%) each for 3 minutes.

Colloidal developer solution was made by dissolving 2 g of gelatin powder (Agar LTD) in 100 ml of double deionized distilled water (2% w/v). This was added to 1% aqueous formic acid. Developer solution was mixed 1:2 volumes with 50 g/dl aqueous freshly prepared double deionized silver nitrate (M & B) solution filtered through mini-pore filter paper under dark room conditions.Histological sections were left in silver colloid solution for 45 minutes at 37°C in an air incubator. Background stain was reduced through holding the slides perpendicularly in Coplin's jars where the precipitate remains at the bottom.Sections were washed in running double deionized distilled water for 10-15 minutes then treated with 10% nitric acid solution (Fluka) for 30 seconds, washed well with flowing double deionized distilled water and immersed in 5% sodium thiosulphate (AnalaR) (w/v) solution for 5 minutes to provide a permanent preparation.Finally, dehydration was achieved by ascending concentrations of ethanol alcohol (70%, 80%, 90% and 100%), each for 3 minutes, then clearing with xylene and mounting with Eukitta mounting medium.

Examination of 45 sections (5 sections/ age group) was done under light microscope

(1250X oil immersion).Five fields showing the region of VNper section were examined and simple random sampling of 20 cells in each field was done for the number of Ag-NOR dots per cell (Figure 1). Hundred cells of VN with Ag-NOR stained nuclei were recorded in each section and the average number of staining dots per each cell was obtained[14].

Results

A view of the developing Rhombencephalon is seen in figure1 that shows the fourth ventricle roofed by the dorsal metencephalon and the medullary velum. The dorsal metencephalon is the site where the cerebellum develops, and it is lined by VN.

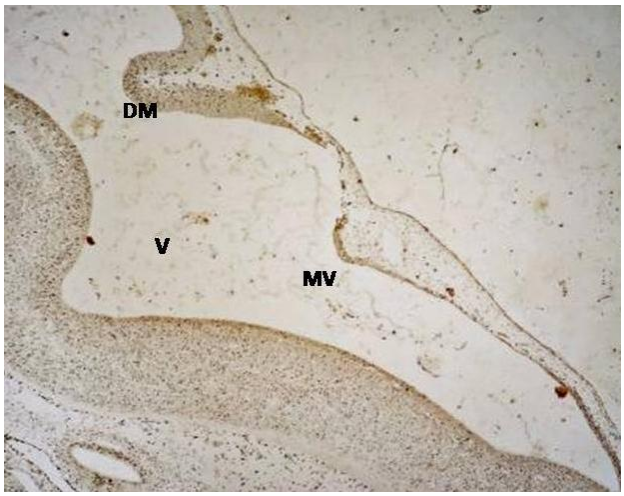

Figure 1.Rat embryo at day 15.Aparasagittal section in the developing Rhombencephalon showing the fourth ventricle (V) roofed by theDorsal Metencephalon (DM) and the Medullary Velum (MV). Paraffin, Ag-NOR stain. 40 X.

Another orientation view of the developing cerebellum is seen in figure 2 that shows a compact cellular layer at the VN that lines the cerebellar primordium. Purkinje cells are observed as a less packed stratum deep to the External germinal layer that spreads from the region of the Rhombic lip to cover the surface of the developing cerebellum, while fronds of cellular projections from the medullary velum mark the development of the choroid plexus at the roof of the fourth ventricle.

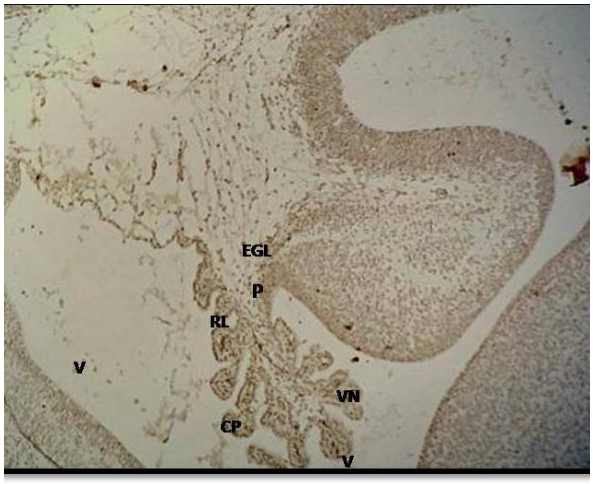

Figure 2.Rat embryo at day 17. A parasagittal section in the developing Cerebellum showing the Ventricular Neuroepithelium (VN), the Purkinje cell layer (P), External germinal layer (EGL), Rhombic lip (RL), Choroid plexus(CP), and the fourth ventricle (V). Paraffin, Ag-NOR stain.100X.

In figure 3, the cells of VN are magnified to reveal the Ag-NOR dots within the nuclei.

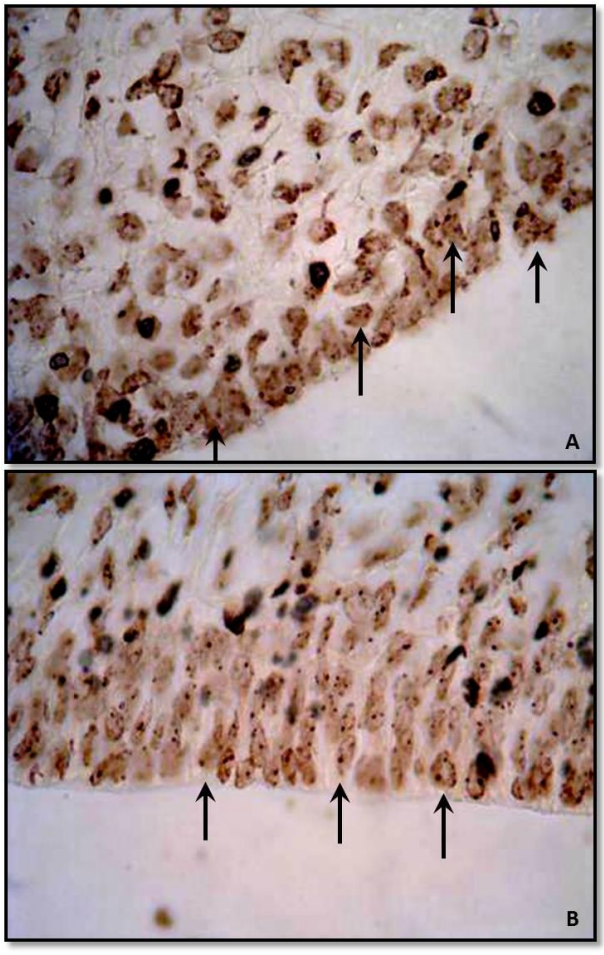

Figure 3. Cells of the ventricular neuroepithelium of the roof plate of the fourth ventricle with maximum mitotic activity at E15 (A) and minimum mitotic Activity at E18 (B) reflected by the number of "dots" (arrows) in the proliferating cells.Paraffin, Ag-NOR stain. 1250 X Oil immersion.

The Average number of Ag-NOR dots per cell nucleus for 100 cells within five fields per age section of VN in nine different age groups of rat embryo are shown in table 1. The results showed an increment in the average numbers of Ag-NOR dots per cell nucleus during E13,E14 to reach its maximum on E15 then they decline on E16-E18 to show another increment again on E19 till birth.

In order to analyze the differences between the various age groups, a single factor ANOVA was applied regarding the Ag-NOR parameter evaluated: the mean Ag-NOR number per cell. The results show a statistically significant difference between the various age groups studied ($P<0.01$).

Table 1. Average numbers of Ag-NOR dots per cell nucleus for 100 cells within five fields per age section of the ventricular neuroepithelium of the roof plate of the fourth ventricle in nine different age groups of rat embryos.

section	Age (Days)								
	E21	E20	E19	E18	E17	E16	E15	E14	E13
1	2.93	2.81	4.79	2.74	2.81	2.83	5.88	5.3	3.32
2	2.54	3.32	3.87	2.4	2.23	2.98	6.47	6.12	4.12
3	2.89	3.1	4.43	2.34	2.88	4.16	7.04	4.71	3.65
4	2.21	3.22	3.98	1.94	3.1	3.13	7.12	5.55	2.98
5	3.1	2.91	4.14	2.62	2.45	2.64	6.64	4.91	3.34
Mean	2.73	3.07	4.24	2.41	2.69	3.15	6.63	5.32	3.48
± SD	±0.36	±0.21	±0.37	±0.31	±0.35	±0.59	±0.5	±0.56	± 0.43

Discussion

Different methods (lectinhistochemistry[18], short-term and long-term survival thymidine autoradiograms[19], enzyme histochemistry [2], and immune histochemistry[9])have studied VNof the developing cerebellumin terms of cellular production and migration. These studies have shown that this region is a germinal zone that undergoes temporal variation in the production of different types of cells that contribute to the cerebellar neurons; none of these have studied the proliferative activity of the cells of this region throughout the prenatal development of the rat CNS.

Many studies performed on various regions of the CNS using the Ag-NOR technique alone or in combination with other histological stains have revealed quantitative associations between cell proliferation and different aspects of functions [12], neoplastic changes [13, 15], and cell production [16].

The results showed a significant increment in cell proliferative activity in terms of Ag-NOR dots per cell nucleus during the embryonic days E13, E14 to reach its maximum in the embryonic day E15. Such results conform to observations made by other birth dating studies indicate that all projection neurons are generated between E13 and E15 in the developingcerebellum of the rat[20, 21].

Previous studies have found that the neurons of the deep cerebellar nuclei are generated from the layer of VN between embryonic days (13-15) with peak production on day 14. The Purkinje cells are also generated from the layer of the VN between embryonic days (13-16) but with a peak on day 15 [22, 23]. The peak of proliferative activity noticed on E15 in this study agrees with the peak of purkinji cells' production noticed on E15 by the previous studies, but it's not the case with the deep cerebellar nuclei.

The results of this work support the results of the recent studies demonstrating that only the GABAergic component of the DCN (neucleo-olivary projection neurons and the

interneurons) are derived from VN, while the glutamatergic components are derived from the rhombic lip [24,25,26].

The proliferative activity of VN cells is seen to be increased again during the embryonic days E19-E21. This period of embryological development is the time of GABAergicinterneuronsproduction (interneurons of the DCN, stellate, basket, Lugaro, and Golgi cells) that are generated during late embryonic and postnatal development [9, 20, 25].

The proliferative activity of VN cells in the early stages of cerebellar formation (E13-E15) is shown to be higher than in the late embryonic stages (E19-E21).Recent studies have revealed that the two main classes of GABAergic neurons are generated in VN according to distinct strategies. Theprojections neurons are produced within VN at the onset of cerebellar neurogenesisand are committed to their fate at early ontogenetic stages. In contrast, inhibitory interneurons derive fromsingle pool of multipotent progenitors that delaminate from the VN during late embryonic life and continue to divide in the prospective white matter up to postnatal development[24, 27].

This work demonstrates that the temporal variation in the proliferative activity of the cells at VN of the developing cerebellum coincides with the birth-dating timetable of the different types of cells produced in this region.

In conclusion, the Ag-NOR staining technique provides a quantitative index of cell proliferation at the ventricular neuroepithelium of theRhombencephalon roof plate to support previous qualitative cellular production and birth-dating studies.

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