Iraqi JMS

Published by Al-Nahrain College of Medicine
ISSN 1681-6579
Email: Iraqi_jms_alnahrain@yahoo.com
http://www. colmed-nahrain.edu.iq/

The Proliferative Profile of the Rhombencephalicdemilune in the Developing Rat Cerebellum: A quantitative HistochemicalStudy

Ali HA Al-Anbaki *MSc*, Huda R Kareem *MSc*, Muthanna AA Al-Kaabi *PhD*

Dept. of Human Anatomy, College of Medicine, Al-Nahrain University

Abstract

Background Studies have shown that the cerebellum develops within the dorsal metencephalon creating a rhombencephalicdemilune (RD) which represents the formation site of the cerebellum granular cells progenitors. These studies used different histological techniques but all have provided qualitative information regarding the biosynthesis and cell mitosis at the RD.

Objective Quantifying the proliferative activity of the cellsat the RD during the embryonic period.

Methods Six age groups from day 16 to day 21 albino rat embryos Rattusrattusnorvegicus were investigated with Ag-NOR staining technique to quantify cell proliferation.

Results There wasa statistically significant difference ($p<0.01$) between cellular activity at different age groups with a surge during embryonic day 18.

Conclusions Correlation with other studies revealed that Ag-NOR staining technique, which reflects protein biosynthesis and nuclear mitotic activity, provided a valuable quantitative measure of cellular proliferation in the developing rat cerebellum.

Keywords Rhombencephalicdemilune, Rat, Developing Cerebellum, Ag-NOR, Quantitative, Histochemistry

Introduction

The cerebellar primordium was described as a crescent shaped anlage or cluster of cells that appear at embryonic day 13. This primordium is divided into three parts: lateral, subisthmal and postisthmalprimordia, which were believed to be the sources of different components of the developing cerebellum [(1)]. Later studies gave a morphological delineation of the developing cerebellar primordium as being a dorsal metencephalic anlage since the metencephalon has floor and roof plates, and the cerebellum develops within the roof plate or the "dorsal metencephalon"[(2)]. Thus, the rhombencephalicdemilune(RD) is the crescent-shape area surrounding the dorsal metencephalic anlage caudally and laterally, forming the caudal part of the roof plate of the forth ventricle in the developing embryo; it is the zone where the progenerators of the cerebellar external germinal layer(EGL) cells are located. These cells spread radially from caudal to rostral direction during the embryonic day 17,which signals the transformation of the cerebellar primordium into the primitive cerebellum [(2,3)].

These events weredemonstrated by different histochemical methods includingshort-term and long-term survival thymidine autoradiograms [(4)], lectins [(5)], immune histochemistry[(6)], and enzyme histochemistry [(7)].Such methodsuse different markers for migration, histological differentiation and cell process dynamics, in particular during complex embryonic development at the dorsal metencephalic anlage[(1,8,9)]. All these methods only provide subjective information regarding the proliferative profile of the RDthat has

specific spatiotemporal variation during prenatal development; it appears at embryonic day 17 and disappears at day 21 [2,10,11].

In the proliferating cells, nucleolar organizer regions (NORs) are loops of DNA which contain ribosomal RNA genes important for the synthesis of proteins. These NORsare stained with silver colloid technique, and the resultis known as Ag-NOR dots [12].

The number and area of Ag-NORs are an accurate index of activity and cell proliferation in terms of protein synthesis[13]. Hence, Ag-NORstain is used to measure the biosynthetic profileand cell mitotic activityby demonstrating the amount of rRNA that increases during cell replication [14]. It can be used as an indicator related to the proliferative capacity of normal and neoplastic cells [15].This work aims at assigning a quantitative proliferation index for the cells of the RDduring their embryonic development by the application of Ag-NOR staining technique.

Methods

A sample of twelve albino rats *Rattusrattusnorvegicus* was divided into six age groups from embryonic day 16 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 16 through day 21.

Sections were stained according to the method of Ploton [16]. 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 sections was done under light microscope (1250X oil immersion) using a systemic random selection of 5 fields per section of each age group.Fifty cells with Ag-NOR stained nuclei were identified in each field and the average number of staining dots per each cell was obtained [14].

Results

An orientation view is seen in figure 1 that shows a compact cellular layer at the RD region with extensions towards the EGL and the neuroepithelial layer. Purkinje cells are observed as a less packed stratum deep to the EGL, while fronds of cellular projections from the medullary velum mark the development of the choroid plexus at the roof of the forth ventricle.In figure 2, the cells of the RD are magnified to reveal the Ag-NOR dots within the nuclei.The averages of Ag-NORs per cell nucleus in different age groups of rat embryo RD are shown in table 1.

With the aim of analyzing the differences between the various age groups, a single factor ANOVA was applied regarding the Ag-NOR parameter evaluated: mean Ag-NOR number

per cell.The results show a statistically significant difference between the various age groups studied (P<0.01), as shown in table 2.

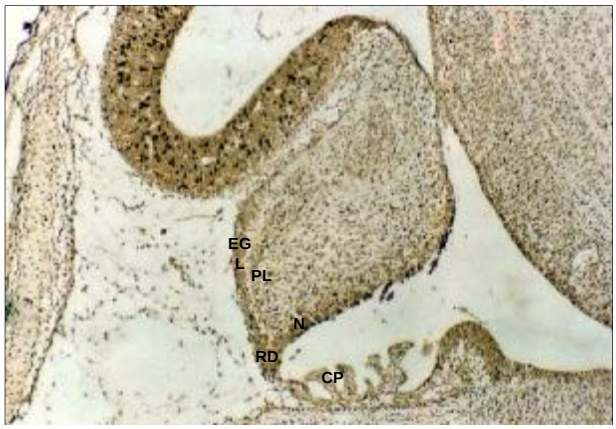
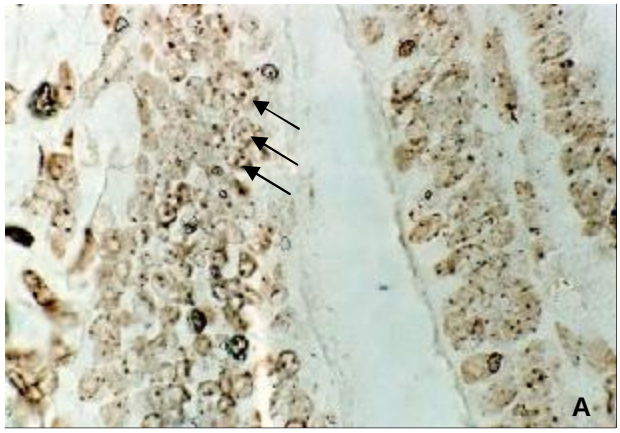
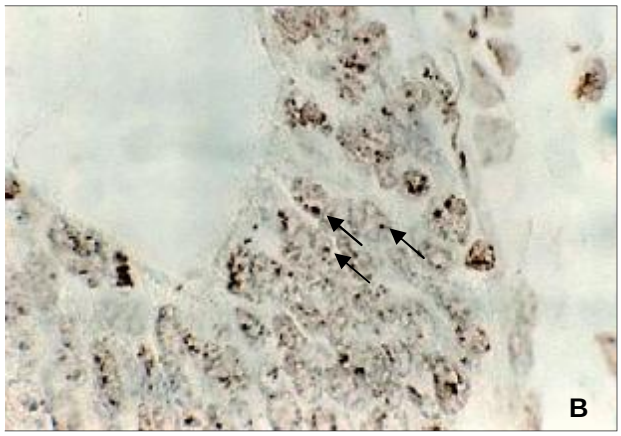


Figure 1. Parasagittal section of the developing cerebellum of a18 days aged rat embryo showing the rhombencephalicdemilune (RD) with the appearance of the cerebellar external germinal layer (EGL), Purkinje layer (PL) and neuroepithelium (N). The choroid plexus (CP) is seen at the roof of the 4th ventricle. Ag-NOR stain. 100X.





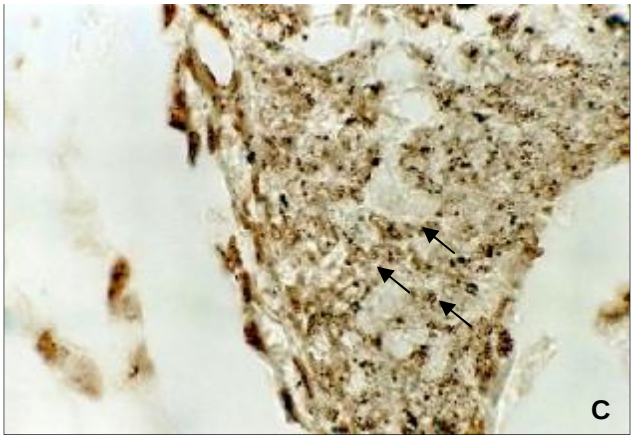


Figure 2.Cells of the rhombencephalicdemilune have increasing mitotic activity from day 16 (A), day 17 (B) with maximum at day 18 (C) reflected by the increasing number of Ag-NOR "dots" (arrows) in the proliferating cells. Ag-NOR stain. 1250X Oil immersion

Table 1. Average numbers of Ag-NOR per cell nucleus identified within five fields of each section in different age groups of rat embryo rhombencephalicdemilune

Section	Age (days)					
	16	17	18	19	20	21
1	1.64	2.74	7.74	1.56	1.40	1.32
2	1.44	2.40	6.92	1.60	1.36	1.06
3	1.62	2.34	6.86	1.64	1.32	1.28
4	1.58	2.88	6.72	1.62	1.38	1.40
5	1.38	2.62	7.02	1.66	1.41	1.24
Mean ±S.D	1.53± 0.12	2.60± 0.23	7.05± 0.40	1.62± 0.04	1.37± 0.04	1.26± 0.13

Table 2. ANOVA Single Factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	126.1153	5	25.22306	622.0748	1.46E-24	3.89507
Within Groups	0.97312	24	0.040547			
Total	127.0884	29				

Discussion

Several works designate the RD as the birth place of the EGL of the cerebellum; this region undergoes cellular changes in correlation with the spatiotemporal variations that results in the spread of the EGLto cover the outer surface of the cerebellum during development[1,2].

The proliferative activity of the RD studied previously gave a clue that the caudal part of the dorsal metencephalic anlage is the formation site of the granular cells progenitors in the EGL [2,3]. But whether designated as a superior [11] or upper rhombic lip [17], the observations made on this formation site were based on qualitative measures performed to characterize the proliferating cells of the region.

In order to investigate the possibility of establishing a quantitative method for the estimation of cellular activity at the RD during embryonic development, the Ag-NOR staining technique was employed in this work because it is considered as a rapidand easy way to obtain an estimation of protein synthesis rate [13,18];the number of Ag-NOR dots is an accurate index of activity and cell proliferation in terms of protein synthesis [19,20].

Although studies performed on various regions of the CNS, such as the hypothalamus and the hippocampus, did not analyze the proliferation pattern seen during the various developmental stages, the Ag-NOR technique alone or in combination with other histological stains have revealed quantitative associations among cell proliferation and different aspects of functions in animals and in humans [21-23].

Our results showed a significant "surge" in cell proliferative activity in terms of Ag-NOR dots per cell nucleus during the embryonic day 18 (Table 1). Such results conform to observations made by other studies when the nuclear transitory zone starts to appear at the embryonic day 14 and the cortical transitory zone at the embryonic day 15 [1].Further dynamic re-arrangement and translocation of the cells at these zones during the embryonic day 16 and thereafter is in the direction of the EGL dispersion, as noticed in figure 1 which demarcatesthe extension of RD cells towards the newly formed EGL. This dispersion, beginning at the embryonic day 17, signals the transformation of the cerebellar primordiuminto the primitive cerebellum, and it is coupled with the progressive maturation of the cerebellar cortex and the deep nuclei [8,9]. Consequently, this study revealed that the proliferative activity of the cells of the RD reaches its peak one day after the appearance of granular cells progenitors when cell "migration" is at maximum [24].

In conclusion, the Ag-NOR staining technique was successfully used in this study to yield a quantitative index of cell proliferation at the RDin concordance with previous qualitative works. This technique is recommended for the investigation of protein biosynthesis and cellular activity in the developing CNS.

References

1. Altman J, Bayer SA. Embryonic development of the rat cerebellum. I. Delineation of the cerebellar primordium and early cell movements. *J Comp Neurol*, 1985 Jan; 231(1): 1-26.
2. Al-Salihi AR. Some aspects of the developmental dynamics and histogenesis in the dorsal metencephalicanalage. Baghdad: Al-Nahrain University, Ph.D. Thesis, 1995.
3. Al-Anbaki A. The Role of the RhombencephalicDemilune of the Dorsal Metencephalic Anlage in the Development of the Cerebellum. Baghdad: Al-Nahrain University, MSc Thesis, 2006.
4. Sack HJ, Stohr M, Schachner M. Cell type-specific binding of Ricinuslectin to murine cerebellar cell surfaces in vitro. *Cell Tissue Res*, 1983; 228(1): 183-204.
5. Altman J. Morphological development of the rat cerebellum and some of its mechanisms. In: Palay SL, Chan-Palay V, editors. The Cerebellum-New Vistas. New York: Springer, 1982; p. 8-46.
6. Yoshioka T, Tanaka O. Histochemical localization of Ca2+, Mg2+-ATPase of the rat cerebellar cortex during postnatal development. *Int J DevNeurosci*, 1989; 7(2): 181-93.
7. Dontenwill M, Roussel G, Zanetta JP. Immunohistochemical localization of a lectin-like molecule, R1, during the postnatal development of the rat cerebellum. *Brain Res*, 1985 Jan; 349(1-2): 245-52.
8. Altman J, Bayer SA. Embryonic development of the rat cerebellum. II. Translocation and regional distribution of the deep neurons. *J Comp Neurol*, 1985 Jan; 231(1): 27-41.
9. Altman J, Bayer SA. Embryonic development of the rat cerebellum. III. Regional differences in the time of origin, migration, and settling of Purkinje cells. *J Comp Neurol*, 1985 Jan; 231(1): 42-65.

10. Altman J, Bayer SA. Development of the precerebellar nuclei in the rat: I. The precerebellar neuro epithelium of the rhombencephalon. *J Comp Neurol*, 1987 Mar; 257(4): 477-89.
11. Williams PL, Banister LH, Berry MM, Collins P, Dyson M, Dussek JE, et al. Gray's Anatomy. 38 ed. Edinburgh: Churchill Livingston, 1999; p. 242.
12. Egan MJ, Crocker J. Molecular biology and respiratory disease. 3. Evaluation of nucleol arorganiser regions in pulmonary pathology. *Thorax*, 1990 Mar; 45(3): 225-32.
13. Garcia-Moreno LM, Cimadevilla JM, Gonzalez-Pardo H, Arias JL. Functional differences between dorsal and ventral hippocampus revealed with AgNOR staining. *Psicothema*, 2000; 12(2): 293-95.
14. Trere D. AgNOR staining and quantification. *Micron*, 2000 Apr; 31(2): 127-31.
15. Al-Shamma'a ZM. Placental trophoblastic disease: Immuno-histochemical stains and AgNORs study. Baghdad: Al-Mustansiriya University. Thesis for Fellowship of Iraqi Commission of Medical Specialization in Pathology, 2001.
16. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. *Histochem J*, 1986 Jan; 18(1): 5-14.
17. Alcantara S, Ruiz M, De Castro F, Soriano E, Sotelo C. Netrin 1 acts as an attractive or as a repulsive cue for distinct migrating neurons during the development of the cerebellar system. *Development*, 2000 Apr; 127(7): 1359-72.
18. Crocker J. Molecular and biochemical aspects of interphase nucleolarorganiser regions. *Clin Mol Pathol*, 1996 Feb; 49(1): M8-M11.
19. Derenzini M, Farabegoli F, Trere D. Relationship between interphase AgNOR distribution and nucleolar size in cancer cells. *Histochem J*, 1992 Dec; 24(12): 951-6.
20. Ploton D, Visseaux-Coletto B, Canellas JC, Bourzat C, Adnet JJ, Lechki C, et al. Semiautomatic quantification of silver-stained nucleolar organizer regions in tissue sections and cellular smears. *Anal Quant CytolHistol*, 1992 Feb; 14(1): 14-23.
21. Fushiki S, Kinoshita C, Nagata A, Tsutsumi Y, Nishizawa Y. A combined staining method for argyrophilic nucleolar organizer regions and for glial fibrillary acidic protein in astrocytes of human brain. *Histochem J*, 1996 Jul; 28(7): 523-30.
22. Gonzalez-Gonzalez S, Gonzalez-Pardo H, Vallejo G, Arias JL. Functional sexual differences in rat mammillary bodies: a quantitative Ag-NOR study. *Acta Anat (Basel)*, 1996; 157(3): 205-9.
23. Garcia-Moreno LM, Cimadevilla JM, Gonzalez Pardo H, Zahonero MC, Arias JL. NOR activity in hippocampal areas during the postnatal development and ageing. *Mech Ageing Dev*, 1997 Aug; 97(2): 173-81.
24. Sanes DH, Rch TA, Harris WA. Development of the Nervous System. New York: Academic Press, 2000; p. 94.

Correspondence to: Dr. Muthanna Al-Kaabi
E-mail: muthakabi@yahoo.com
Received 13th Feb. 2011: Accepted 20th Jun. 2011