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Laminin Expression Pattern in the Histogenesis of the Dorsal Metencephalic Anlage

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Abstract: ***Although laminin is the first glycoprotein to be expressed during embryogenesis of the CNS and it is involved in the list of the fifty proteins responsible for recessive cognitive disorders in human. But its importance in the cerebellar development has not received much attention. This study is an attempt to shed light on the functional role of the laminin in the development of one of the key structures in the central nervous system. That is the cerebellum in order to clarify its therapeutic potential in the treatment of these disorders. The mammalian animal model of this study was the rat and the embryonic period covered in it,was from the embryonic day 15 till birth. E-designation system was used to determine the relevant developmental stages of (62) rat embryos. Embryonic samples were collected and processed for paraffin block then sectioned. Polyclonal anti laminin Ab was used to demonstrate laminin reactivity. Quantification of laminin Ab reactivity done using Aperio Alogrithm software. The results of the present study revealed that the laminin was present at the pial basement membrane delineating the dorsal surface of the developing cerebellum and concentrated at the fissures. It illustrated that the spatiotemporal variation of the laminin expression is correlated with the main cellular dynamic events occurring in the dorsal part of the metencephalon. This work concluded that lamininisa navigation cue for migration, proliferation, differentiation, neurite outgrowth and cell process dynamics in particular during complex embryonic development taking place in the dorsal metencephalic anlage.***

Keywords: Dorsal metencephalic anlage, germinal trigone,external germinal layer, settlement of Purkinje cells layer.

1. Introduction

Rhombencephalon is the primordium of the adult hindbrain which represents the region of the neuroaxis that surrounds the fourth ventricle and its recesses. It is subdivided into two parts, the caudal part (myelencephalon) and the rostral part (metencephalon) by the pontine flexure. The myelencephalon becomes the medulla oblongata, whereas the metencephalon becomes the pons and cerebellum. The cavity of the rhombencephalon becomes the fourth ventricle and the central canal in the medulla. The basal plate; ventral portion of the rhombencephalon is continuous with spinal cord caudally and with tegmentum of mesencephalon by a narrowed isthmus region rostrally. This continuity with the caudal and rostral neuroaxis is absent dorsally. The alar plate; the dorsal part of the rhombencephalon fails to fuse medially in this region. Instead, a membrane, the medullary velum, part of which becomes the tela choroidea, spreads over the fourth ventricle. This membrane initially forms a simple covering over the fourth ventricle and inter connects the edges of the caudal and the rostral portions of the classical dorsal rhombencephalon. The bridgeheads are the dorsal metencephalon rostrally and the pre-cerebellar neuroepithelium caudally[1].The cerebellum develops “in” the dorsal metencephalon[2].Different authors gave different delineations and morphological descriptionsto the roof plate of the developing rhombencephalon such as; cerebellar anlageand dorsal metencephalic anlage (DMA) [3].During cerebellar development,two germinal zones appear in the DMA; ventricular zone (VZ) and rhombic lip (RL).VZ is responsible for generations of all GABAergic cerebellar neurons (deep nuclei neurons include nucleo-olivary neurons, Purkinje cells and Golgi while RL is responsible for generations of all glutamatergic cerebellar (include granular cells, unipolarbrush cells and excitatory projection neurons) and several hindbrain neurons. These neurons after their generation migrate to their final destination[4]. As a result to that, major dynamichistogenetic events take place in the DMA such as formation of the primitive cortex, external germinal layer, development of germinal trigone and migration of the Purkinje cells[5].

Two types of migration; radial and tangential migration are reported during the developing cerebellum in the early development[6].In radial migration, migrating Purkinje cells move outward in course, perpendicular to the ventricular surface along the Bergmann cells whose fibers serve as a scaffolding for migrating cells (radial guided movement). Bergmann glia are specific radial glia located in the developing cerebellum in early developmentand play a critical role in the migration of the cerebellar Purkinje cells and granule cells[7].In contrast to the radial migration, migrating PG cell in tangential migration move in trajectory, parallel to ventricular surface and independent to the glial guidance away from the rhombic lip due to the combined effect of chemorepellent and chemoattractant cues[8].Several extracellular matrix (ECM) components are essential to control these dynamic events. Of these, is the laminin[9].

Laminins include a group of the heteromeric ECM glycoproteins consisting of α, β, and γ chains. They have been implicated in many biological functions such as cells differentiation, cells adhesion, cells migration and neurite outgrowth[10]. Laminins are essential for accurate cerebellar development and are vitalfor proper confirmation of meningeal basement membrane and for proper Bergmann glia development[11]. Moreover, laminin deficiency causes a

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decrease in the proliferation and migration of the granule cell progenitors, disorganization of Bergmann glia cells[12].

2. Materials and Methods

2.1 Experimental animals and housing

The present study was conducted on thirty albino mature female rats (Rattusrattusnorvegicusalbinus), they were selected on basis of being obviously active and healthy, with 280 ± 30 g body weight. The female rats were housed separately in a controlled room temperature (25±2o°C) on 12 hr light/dark cycle (lights on 7.00 am) with free access to fresh trefoil diet and water ad libitum. They were placed in poly propylene cages with three animals per cage and were permitted to acclimatize with the laboratory conditions three weeks. The isolated female was daily examined to segregate those in estrus.

Mating the animals and timing of pregnancy
The females at estrus time were put in separate cages for breeding, each two females with one mature male and left overnight. The females were examined early in the next morning between 8:00-9:00 am. In this work, the gestational day zero was defined as the day when a copulatory plug and/or spermatozoa in a smear of the vaginal contents were observed in situ. The female was then transferred to a separate cage and chronologically labeled for day post coitum (dpc) which is a day that follows the day of copulatory plug observation.

Retrieved of Embryosand tissue Preparation:
At the appropriate post copulatory age, the pregnant females were anaesthetized by deep anaesthesia with chloroform (Ajax chemical) in an air-tight jar for 15 minutes then the animals were pinned in supine position and transverse abdominal incision was performed then the two cornua of the uterus were dissected out. The embryos were extracted from the gestational sacs and the extra-embryonic membranes were removed, rinsed in normal saline, then each embryo was carefully examined under the dissecting microscope. After that, two embryos for each post copulatory age were transferred to 10% neutral buffered formalin for two week for the Crown-Rump Length (CRL) measurement [13, 14]and the 15, 16 & 17 days old embryos (E15-17) were decapitated and only the head was fixed in 10% neutral buffered formalin for 36 hours. Embryos 18 days old and more(E18-21)were first decapitated, and the head was fixed in 10% neutral buffered formalin for 24 hours then under dissecting microscope, the calvaria was removed, and the hindbrain was mobilized and delivered out by transecting the tectum of the mesencephalon. Only the hindbrain was immersed in the 10% neutral buffered formalin for 24 hours. After fixation, the samples were ready for histological preparation of dehydration, clearing, paraffin embedding, sectioning dewaxing and hydration. All of them were done according to (Bancroft*et al.*, 2013).[15]

Determination of the Chronological Age
No standard development staging system for rodent embryos was found. Investigators chose a varieties of systems that differ significantly[16]. In the current study, we used the E-designation system[3]in order to standardize the embryological material, depending on the previous scientific developmental staging systems. This system uses the letter E referring to a specific developmental stage of the rat embryo followed by a numerical value which refers to embryonic day. Although this designation is very close to postcopulatory age, but it is not identical with it. In order to detect the exact chronological age of the embryos, several parameters are included in this system such as postcopulatory age, CRL, Theiler's stage and Carnegie's stage.

Immunohistochemistry
We used enzyme labeled antibodies technique for visualization of laminin. Rabbit polyclonal anti-Laminin primary antibody (catalog # NB300-144) from Novus Biologicals® and the Super Sensitive IHC for Detection Kit (catalog # orb219874) from Biorbyte® were used. The sections were processed at room temperature in a humidified chamber. Sectioning at 4µ were used and deparaffinized, Incubate tissue in appropriate pretreatment or digestive enzyme for primary antibody, digestive enzyme use (pepsin enzyme); and PBS/TBS wash 3 times for 2 minutes. Then incubate slide in Hydrogen Peroxide Blocking Reagent for 10 minutes, PBS/TBS wash 3 times for 2 minutes. Apply Blocking Reagent and incubate for 5 minutes, PBS/TBS wash 3 times for 2 minutes (May be omitted if primary antibodies are diluted in buffers containing normal goat serum). Apply primary antibody and incubate according to manufacturer's recommended protocol (overnight) incubation, PBS/TBS wash 3 times for 2 minutes. Apply HRP Polymer and incubate for 10 minutes, PBS/TBS wash 3 times for 2 minutes.Add 30 ul (1 drop) DAB Chromogen to 1 ml of DAB Substrate, mix by swirling and apply to tissue. Incubate for about 3 - 5 minutes, PBS/TBS wash 3 times for 2 minutes. Finally counter stain and cover slip using a permanent mounting media.

Assessment of the immunohistochemical stainings
Aperio Positive Pixal Count Algorithm was used to evaluate and quantify the distribution of the laminin in prenatal developing cerebellum. This program quantify the amount of the immunhistochemical stain present in a slide image and it has a set of default input parameters when selected. These inputs have been pre-configured for brown color quantification in the three intensity ranges (weak positive=yellow color, positive=orange color and strong positive=brown color).The staining reactivity had been represented by calculating the mean of the positivity percentage[17].

3. Results

On E16, a weak expression of laminin on the dorsal subpial regions of developing cerebellum was noticed as compared with that in the choroid plexus vicinity. It had a slight brown linear distribution around the blood vessels and meningeal membrane. The weak reactivity to laminin was not only seen on the dorsal surface of the developed DMA but it also noticed in other regions of DMA and in the ventricular neuroepithelium but the intensity was differed. It was found that the mean positivity percentage at dorsal surface of DMA was 0.069±0.8 while at ventricular neuroepithelium was 0.012±0.32, so that it seemed to be there was

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concentration gradient of the laminin crossing DMA from the dorsal surface to ventricle Figure (1) and, Figure (2).

On E17, a period of germinal trigone appearance, a more positively intense extracellular reaction was observed over the caudal part of dorsal metencephalic anlage and spread laterally with less intensity as the mean of positivity percentage was 0.3±1.4 and 0.103± 1.2 respectively. There was feeble reaction in the other regions and negative in germinal trigone Figure (3).

On E18, a period of the appearance external germinal layer (EGL), migration and settlement of Purkinje cells. There was a wide spread of laminin expression with strong positive reaction over dorsal surface of developing cerebellum ahead of the spreading front of EGL with mean positivity percentage was 0.4±1.52. There were also scattered patches of strong positive reaction noticed in the region of migrating Purkinje cells where the mean positivity percentage was 0.045± 0.325 and negative reaction in the region ventricular neuroepithelium Figure (4)

On the successive embryonic days E19- E21, a period of the appearance of the principle fissures and cardinal lobes, fourth features were recognized during the examination the immunohistological stained slides and analysis the result of Aperio program. The first feature was that the pattern of laminin expression was not altered regarding its location where we observed a strong positive extracellular immune reaction delineated the dorsal surface of developing cerebellum and distributed along the line of EGL spread with conspicuous intensification in the location of the fissures. The mean positivity percentage were found to be 0.5±1.581, 0.8±1.27and 0.99±0.94 at E19, E20 and E21respectivity Figure (5), Figure (6), Figure (7), and Figure (8).Secondly, a positive reaction was detected in the region of Purkinje cells migration as compared to that of the EGL and negative reaction was visualized in the neuroepithelium vicinity Figure (9). Thirdly, a presence of caudo- rostral concentration gradient of strong positive reaction along the line of the EGL spread was obvious at E21 where we saw a positive reaction with mean positivity percentage 0.998±0.141 at lobe anterolateral lobe in compared to a strong positive reaction with mean positivity percentage 0.875±0.94 at lobe central lobe Figure (10).Lastly, the dorso- ventral concentration gradient of the immune reaction was persistence along all embryonic days with increment with age where at E21 the mean positivity percentage at the dorsal surface of the PCC was 0.899±0.94 as compared to o.153± 0.67 at the region of Purkinje cells migration Figure (11).

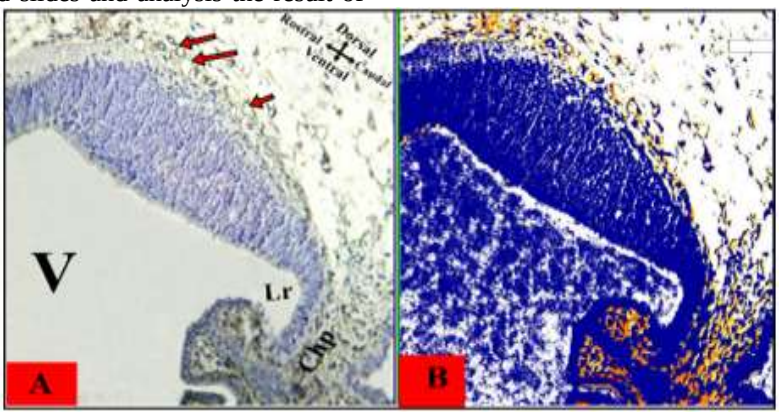


Figure 1: (A) E16, Lateral parasagittal section through DMA: shows weak reactivity of laminin at subpial surface of developing DMA (arrows) ;Chp, choroid plexus; Lr, lateral recess of 4th ventricle, 4th ventricle. IHC (anti-Laminin), 400X.**(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software.

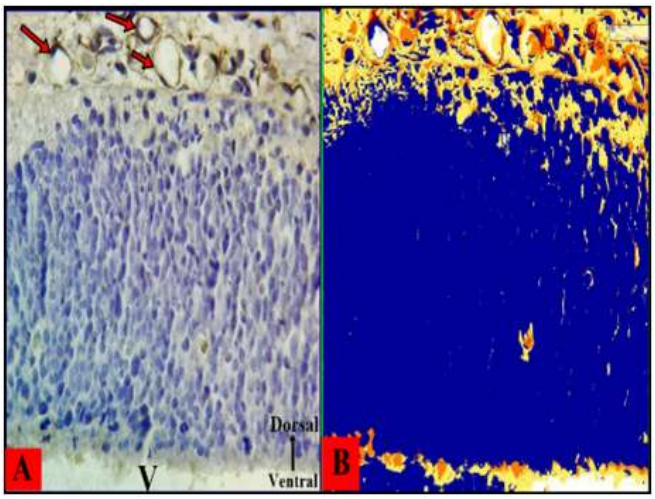


Figure 2: (A) E16, Lateral parasagittal section through DMA: shows weak reactivity of laminin at subpial surface of developing DMA and patches of positive reaction specially around blood vessels(arrows); V, 4th ventricle. IHC (anti-Laminin), 1000X.**(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

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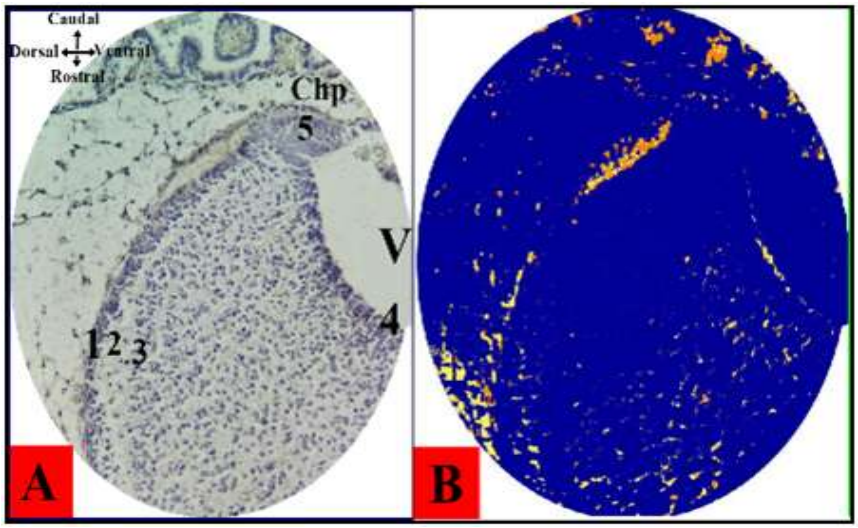


Figure 3: (A) E17, Lateral parasagittal section through DMA: shows positive reactivity of laminin at subpial surface of developing DMA and at a region of migrating purkinje cells ;Chp, choroid plexus; V, 4th ventricle; 1,EGL; 2,primitive molecular layer ; 3, nuclear migrating layer; 4,ventricular neuroepithelium ; 5,germinal trigone. IHC (anti-Laminin), 400X.**(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

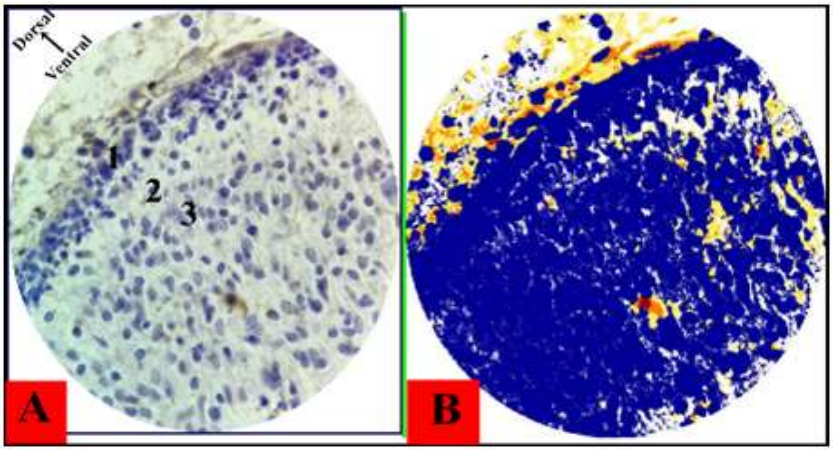


Figure 4: (A) E18, Lateral parasagittal section through DMA: shows positive reactivity of laminin at subpial surface of developing DMA , weak reaction at the region of migration purkinje cells ; 1,EGL; 2,primitive molecular layer; 3,migrating purkinje cells layer ; . IHC (anti-Laminin), 1000X.**(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

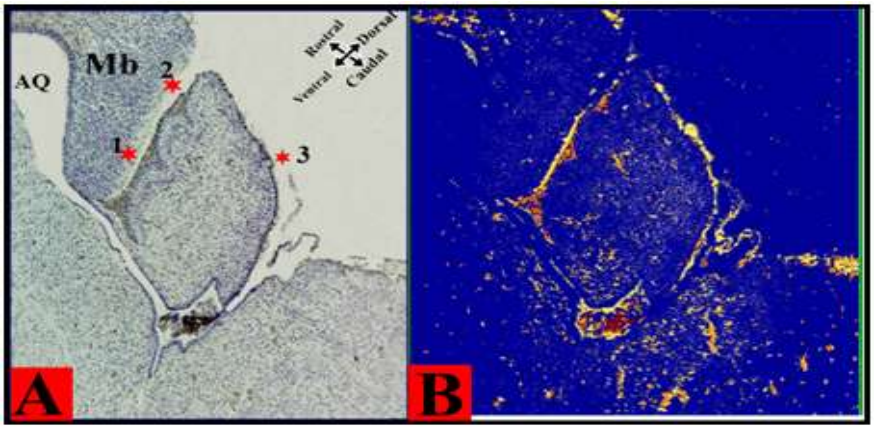


Figure 5: (A) E19, Lateral parasagittal section through DMA: shows positive reactivity of laminin at subpial surface of developing cerebellum and at the region of migration purkinje cells ; **asterisk(1)**,preculminate fissure ; **asterisk(2),**primary fissure ; **asterisk(3)**, secondary fissure ; AQ ,aqueduct ; Mb mid brain ; . IHC (anti-Laminin), 40X.**(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

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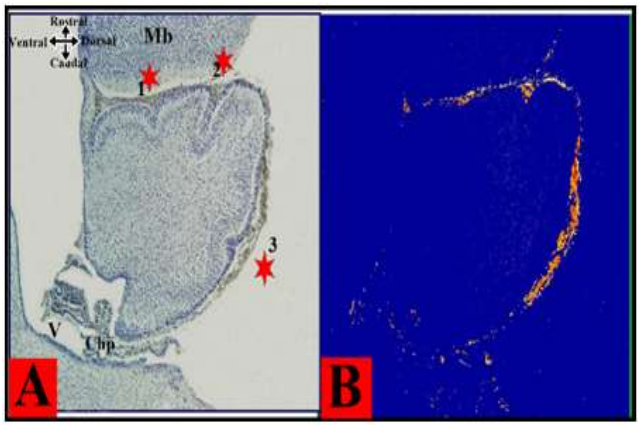


Figure 6: (A) E20, Lateral parasagittal section through DMA: shows positive reactivity of laminin at subpial surface of developing cerebellum.; **asterisk(1),** ,preculminate fissure ; **asterisk(2),** ,primary fissure; **asterisk (3),** secondary fissure; Chp, choroid plexus;V,4th ventricle; Mb mid brain ; . IHC (anti-Laminin), 40X. **(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

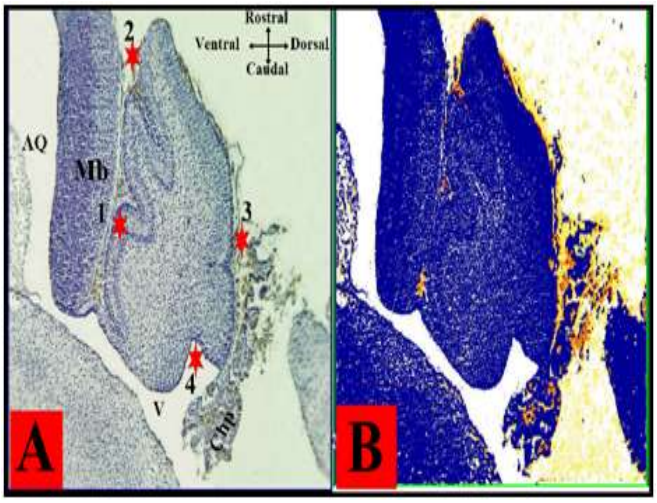


Figure 7: (A) E21, Lateral parasagittal section through DMA: shows positive reactivity of laminin at subpial surface of developing cerebellum.; **asterisk(1)**,preculminate fissure ;**asterisk(2)** ,primary fissure; **asterisk(3)**,secondary fissure; **asterisk (4)**, posterolateral fissure ; AQ ,aqueduct ; Mb mid brain ;Chp, choroid plexus; V, 4th ventricle. IHC (anti-Laminin), 40X. **(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

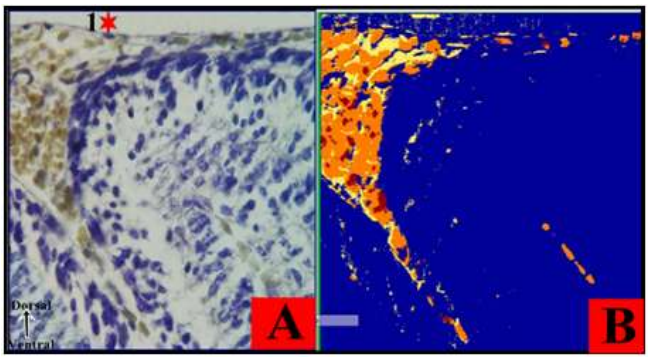


Figure 8: (A) E21, Lateral parasagittal section through developing cerebellum: shows positive reactivity of laminin at subpial surface of developing cerebellum with high intensification at the primary fissure **(asterisk 1)**. IHC (anti-Laminin), 1000X.**(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

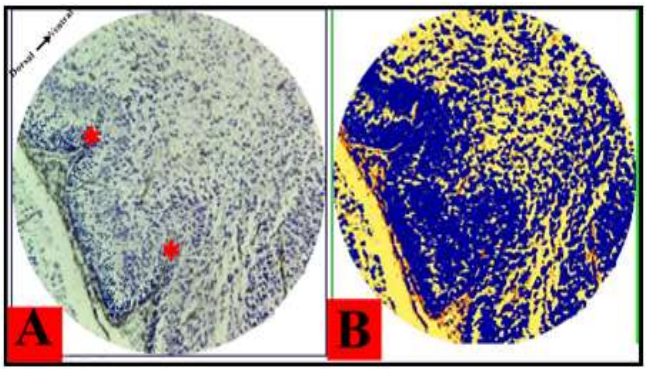


Figure 9: (A) E21, Lateral parasagittal section through developing cerebellum: shows positive reactivity of laminin at subpial surface of developing cerebellum and weak reaction at region of Purkinje cell migration. **Two asterisk** delineate anterolateral lobe. IHC (anti-Laminin), 400X.**(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

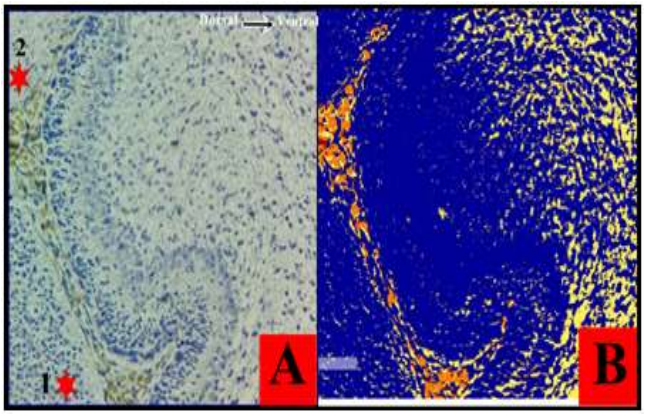


Figure (10): (A) E21, Lateral parasagittal section through developing cerebellum: shows strong positive reactivity of laminin at subpial surface of developing cerebellum with high intensification at preculminate fissure (**asterisk 1**) and the primary fissure (**asterisk 2}**. IHC (anti-Laminin), 400X. **(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

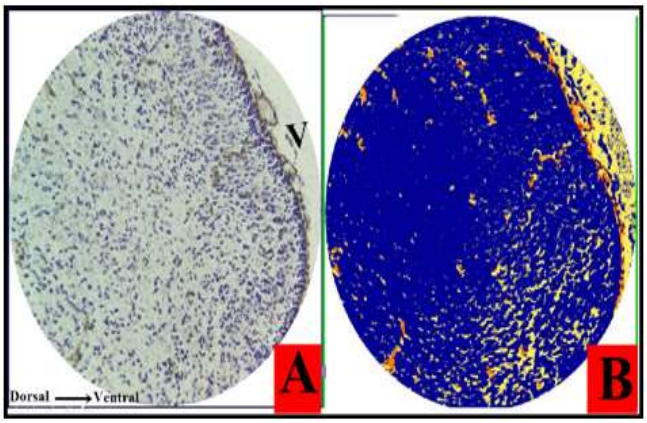


Figure (11): (A) E21, Lateral parasagittal section through developing cerebellum: shows positive reactivity of laminin at subpial surface of developing cerebellum and weak reaction in the migrating purkinje cells region. IHC (anti-

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Laminin), 400X. **(B)** Snap shoot for the section (A) as analyzed by Aperio positive pixel count algorithum software

4. Discussion

Four striking observations were explored in our study. First, the Laminin was strongly expressed in the pial basement membrane delineating the dorsal surface of the DMA and later, the developing cerebellum and in the basement membrane of blood vessels at all stages of the development. It seems that the pial basement membrane is a rich source of the laminin and we suggest it has a role in its integrity. At this point our suggestion is match with that of (*Miner and Yurchenco, 2004; Willi et al., 2002*)[18,19]who reported that the Laminins are not required for basement membrane assembly only, but they are also required for the regulation of its cellular behavior through interactions with cell surface receptors, including integrins, α- dystroglycan, and syndecans . (*Edwards et al., 2010*)[20] reported that the basement membrane is responsible for efficient localization and anchoring of radial glial cell endfeet. Moreover, the survival of the radial glial cell depends on the signals from the meninges[21]. In the developing cerebellum, newly born neurons use Bergmann radial glia as scaffolds, traveling along their fibers in order to reach their final destinations (glia guided migration). Bergmann glia, specialized radial glia present transiently in the cerebellum during its development and send their apical processes to the subpial surface to anchor through interactions of their cell surface receptor, integrin with the pial basement membrane[22]. Integrins, main receptors for the Laminins are abundant along the Bergmann radial glia fibers and are essential for the proliferation and migration of granulecells [23, 24].

Our second noticeable observation in this work was that an apparent spatio-temporal concentration gradient of the expression of the laminin was evident across the subpial surface of the DMA and later the developing cerebellum in a lateral –to- medial direction and then across dorsal surface of the anterior vermis in a caudo- rostral direction. It seems that laminin concentration gradient creates a chemotactic force attracting the migrating cells towards the side of the higher concentration and the laminin may represent an attractive guidance cue for migrating granule cell precursors. So we postulate that the laminin involves in the proliferation and tangential migration of granule cell precursors. In the developing cerebellum, there are two type of the neuronal migrations; radial (glia guided) and tangential migrations[25].The tangential migration characterizes by three features which are; the migrating cells move in a trajectory that is parallel to the ventricle, they exhibitan unipolar morphology and the presence of the gradient of guidance cue[26]. At this point, our results differ from those of (*Shantanu et al., 2014*)[27] since these authors mentioned that the laminin is not involved in the tangential migration of the granule cell precursors. Two explanations account for this disparity. First, these authors used anti-laminin α1 antibodies and there are seven distinct laminins and some cells may express a type which was not recognized by antibodies used by these authors while we used pan-Laminins antibodies in our study. Second explanation for this disparity between the study of (*Shantanu et al., 2014*)[27] and our study is that these authors used a knockout mice as animal model and the development period considered by them were postnatal period (P) (P7 and P20) while we used the rat in our study as animal model and we covered the embryonic period from 15-21(E15-E21) as the period of studying. Our study is in agreement with that of (*Gupta et al., 2010*)[28] who reported that laminin presents in the postmitotic layer of the EGL and acts as an inducer of the sonic hedgehog (SSH) - induced proliferation of the granule cell precursors which is secreted by Purkinje cells.

Our third attracting observation in this work was that an obvious spatio-temporal concentration gradient of the expression of the laminin was evident at all embryonic ages and increased sequentially across the DMA and later the developing cerebellum from the ventricle to the cortical surface in concomitant with changes in the distribution and morphology of the purkinje cells. At E18, the purkinje cells somas were distributed over the DMA, with a higher density below the external germinal layer (EGL) where they settled in multi-layer band. In contrast to the somatic migration pattern, the leading processes of migrating Purkinje cells elongated deeper into the laminin-rich regions of the DMA and later the developing cerebellum. It seems that this laminin concentration gradient may provide cues for the migration of the Purkinje cells and the direction of the axon growth. So we postulate that the laminin involves in the radial migration and neurite outgrowth of purkinje cells. Our results support and expand reports by (*Shantanu et al., 2014*)[27] who mentioned that laminin was essential for Bergmann glial processes and dendritic tree formation of the purkinje cells. The radial (glial guided) migration characterizes by three features which are; the migrating cells move in a trajectory that is perpendicular to the ventricle, they exhibits a bipolar morphology and presence of the guidance cue (attractive or repulsive) [6]. Reelin, an extracellular matrix glycoprotein is secreted by Cajal–Retzius cells (CR cells) and forms a layer on the dorsal surface of the cortex. It promotes the radial neuronal migration of purkinje cells and instructs them to adopt their proper destination in the cortex[29]. Moreover, laminin has epidermal growth factor repeats similar to reelin so laminin may represent a repulsive guidance cue for purkinje cells migration[30].

Our fourth emphatic observation in this work was that the concentration of the laminin expression intensified at the fissures and this intensification was even more prominent in subsequent days (E21) as the developing cerebellum rapidly enlarged and formed lobules in concomitant with changes in the thickening of the EGL and Purkinje cells position at the base of fissures at this embryonic age. We postulate that the laminin has a role in the process of foliation and our result support the reports of (*Céline et al., 2011*)[31] who studied the cerebellar development in the conditional Lama Knockout mice and they noticed a small size cerebellum with reduction in the depth and number of the fissures and marked reduction of the lobular organization.

5. Conclusion

In conclusion, the piameter has a role in the histogenesis of the DMA as its basement membrane is a rich source of the laminin and the coordinated spatio-temporal expressions of

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the laminin is closely associated with the granule cell precursors and Purkinje cells developments, namely tangential and radial migration respectively.Thisorchestrated spatio-temporal expression of the laminin in developing cerebellum suggest their involvement in the directing and formation of the cerebellar neural architecture.

6. Acknowledgment

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