MMP-2 and TIMP-2 markers for immunohistochemical study on the prenatal development of the rat's primary visual cortex

Mustafa Mohammed Ibraheem
Department of Anatomy, Histology and Embryology, College of Medicine, Al-Mustansiriya University-.

الخلاصة:

ان ظهور العديد من الاثار الجينية (البروتينية) في مناطق متعددة من الدماغ مرتبط بالتقدم في العمر والتطور والذي قد يؤدي الى حدوث تغيرات دائمية، لذلك من المهم جدا" دراسة الظهور الطبيعي لمختلف البروتينات لغرض تحديد وربط وظائفها الفسلجية.

الهدف من الدراسة هو ربط التغيرات ماقبل الولادة مع النماذج المكانية والزمانية لظهور فعالية المسبارات المدروسة. ان المحددات الكيميائية والجزيئية للتطور سوف تدرس في محاولة لفهم بعض العوامل المسيطرة على الاحداث التطورية لما قبل الولادة.

ثمانية عشر من أجنة الجرذ قد استخدمت, ستة اجنة لكل عمر جنيني (يوم 16, يوم 18, يوم 20), ثلاثة اجنة من كل فئة عمرية لدراسة المسبار الجزيئي (MMP-2) والثلاثة اجنة الاخرى لدراسة المسبار الجزيئي (TIMP-2).

خلال تطور القشرة الدماغية في فترة ماقبل الولادة, فان المسبار الجزيئي (MMP-2) يكون موجود في القالب الخارج الخلوي في جميع مناطق التطور للقشرة البصرية، بينما لا توجد فعالية للمسبار الجزيئي (TIMP-2) في القالب الخارج الخلوي في أي مرحلة جنينية.

لذا فان تطور القشرة البصرية يتضمن العديد من التغيرات الشكلية وان هذه الدراسة سوف تساعد في تحديد وتثبيت العوامل التطورية المختلفه للقشرة البصرية الاولية للجرذ.

Abstract:

The objective of this study is to relate the prenatal changes with the spatiotemporal pattern of expression and/or activity of the studied markers. The chemical and molecular determinants of the development will be analyzed in a trial to understand some of the factors controlling these prenatal developmental events. Expression of many genes in various areas of the brain is age and development related and may undergo an irreversible change. Therefore, it is very important to study the normal expression of various proteins in order to delineate their physiological functions.

Eighteen embryos were used, six embryos for each embryonic age (E16, E18 and E20), three for studying MMP-2 immunohistochemical marker and

other three for studying TIMP-2 immunohistochemical marker. During prenatal cortical development, the reaction of MMP-2 marker was located within the extracellular matrix of the all developing zones. While no activity or reaction of TIMP-2 could be depicted through out the extracellular matrix at any embryonic age.

The ontogeny of the visual cortex involves many morphogenetic changes and this study will help in determining and confirming the various developmental criteria of the rat primary visual cortex.

Introduction:

The complete lack of sulci in the rat brain does not permit a proper separation of the various neocortical areas into respective lobes as is possible in higher mammals. Conveniently the areas are referred to homologues in the lobated neocortex [1]. The forces that direct the course of development of the complex nervous tissue of vertebrates are largely unknown. The markers can be considered as an oriented microstructure to guide the developing cells and so act as neurobiotaxis. An increasing number of studies provide evidence for a role of immunohistochemical markers in nervous system development [2]. Presently, immunohistochemistry is firmly established as the most important method for detecting antigens with the light microscope. It can be effectively used to examine various antigens in the sections of formaldehyde-fixed and paraffin-embedded tissues. However, the central problem in immunohistochemistry is to retain antigenicity without sacrificing the quality of cell morphological preservation. It has been established that the preservation of antigenicity is inversely related to the preservation of cell morphology [3].

The wall of the earliest cerebral hemisphere, as elsewhere in the neural tube, consists of a pseudostratified epithelium. The cells elongate and their non-nucleated peripheral processes constitute a marginal zone, whilst their nucleated, paraluminal and mitosing regions constitute the ventricular zone. Some of the mitotic progeny leave the mitotic zone and migrate to occupy an intermediate zone. Groups of germinal cells undergo differentiation forming, at first, generations of definitive neuroblasts and, later, definitive glioblasts, which migrate and mature in their final positions.

The earliest migration of definitive neuroblasts from the ventricular and intermediate zones occurs radially until they approach, but do not reach the pial surface, their soma becoming arranged as cortical plate [4].

In over all organization the telencephalon could be divided into a series of layers, named according to Boulder Committee [5], from the pial surface inwards as the following zones: marginal, cortical plate, subplate, intermediate, subventricular, and ventricular.
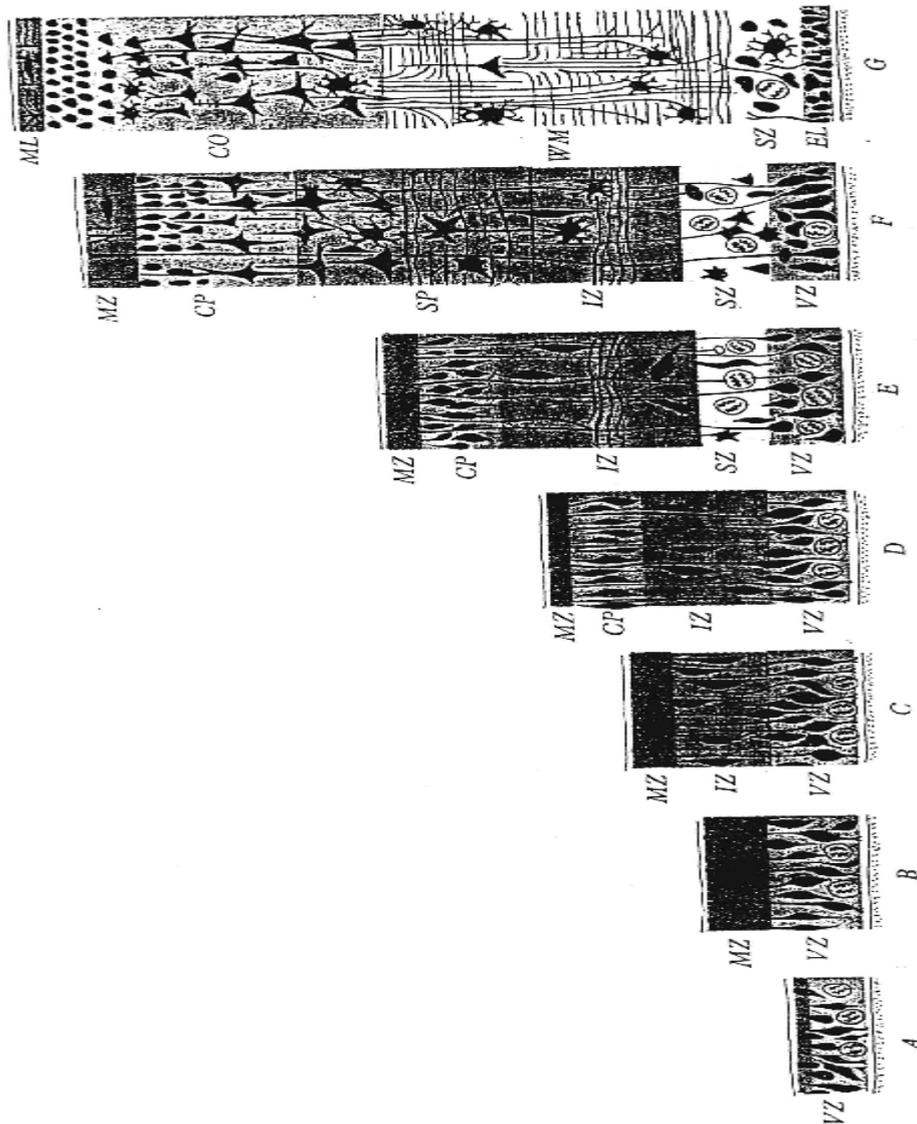
Figure-1: **Formalized diagram of the laminar development of cerebral neocortex based on the original proposals of Boulder Committee (1970):VZ-ventricular zone; MZ-marginal zone; IZ-intermediate zone; CP-cortical plate; SZ-subventricular zone; SP-subplate zone; CO-definitive neocortex; WM-white matter; EL-epindymal layer; ML-molecular layer.**

The marginal zone forms the outermost layer of the cerebral cortex, the neuroblasts of the cortical plate and subplate form the neurons of the remaining cortical laminae, whilst the intermediate zone gradually transforms into the white matter of the hemisphere. Meanwhile, other deep germinal cells have been producing generations of glioblasts, which also migrate into the more superficial layers. As proliferation wanes and finally ceases in the ventricular and subventricular zones their remaining cells differentiate into epindymal cells [6]. Glial cells which stretch radially across the full thickness of the wall of the

telencephalon provide contact guidance paths for the subsequent peripheral migration of groups of functionally related neuroblasts.

A prenatal developmental study by probing the primary visual cortex, in rat's embryo from prenatal (embryonic) day sixteen (E16) till birth (E21) using the following Immunohistochemical markers or techniques for formalin-fixed and paraffin-embedded tissue:

● Matrix Metalloproteinase-2 (MMP-2).

● Tissue Inhibitor of Matrix Metalloproteinase-2 (TIMP-2).

The aim of the study is to relate the prenatal changes with the spatiotemporal pattern of expression and/or activity of the studied markers.

Materials and Methods:

Nine adult female rats (*Rattus rattus norvegicus albinus*) (360 ± 40g body weight) were used. Three females were put in breeding cages at room temperature (22 ± 2ºC) with one male. Food (as standard diet pellets) and water were available *ad libitum.* Females were daily examined before noon, and the dripping tray was inspected. The presence of a vaginal plug was considered as an indication to copulation. Females with a positive vaginal plug were considered to be at day one of gestation.

At the intended postcopulatory age, embryos were recovered. Under chloroform anesthesia, a midline incision was made at the anterior abdominal wall; the uterus was dissected out. The mother was then decapitated. The gestational sacs were incised, two embryos, one from each cornu, were transferred to 10% neutral buffered formalin. So, six embryos for each embryonic age (E16, E18 and E20), three for studying MMP-2 marker and other three for studying TIMP-2 marker. To include an embryo under a particular embryonic age (E), several parameters are observed and should be fulfilled, they include: post-copulatory age, crown-rump length, Theiler's stage and Carnegie's stage. Calvaria was removed by a scissors, the whole brain was scooped out and sectioned so that only the posterior half of each cerebral hemisphere was spared for further treatment.

Serial sections of (10μ) thickness were obtained in the coronal plane for Nissl's stain, and (5μ) were obtained in the coronal plane for immunohistochemical staining technique with mouse anti-human MMP-2 (proform) (Chemicon, USA): Clone: CA- 4001. Isotype: Ms IgG1. Concentration: 200μg/ml. And mouse anti-TIMP-2 (Chemicon, USA): Clone: 3A4. Isotype: IgG_{2a}/K. Concentration: 200μg/ml.

Secondary antibody to each previous primary antibody is from Universal DakoCytomation. This to give high levels of signal amplification due to the binding of multiple units of secondary antibody to the primary antibody.

Since the visual cortex cannot be recognized easily in Nissl preparations of the brains from rats younger than six postnatal days, tissue located in the analogous region of the cerebrum can be considered to be presumptive visual

cortex [7]. So in embryonic stages the caudal part of the developing neopallium was chosen to represent the visual cortex in this study.

Results:

At E16, the wall of the caudal part of the cerebral hemisphere exhibited ventricular, intermediate, and marginal zones. At E18, the migrating neuroblasts of the ventricular zone formed the cortical plate (Fig.2). At E20, the following zones were defined in the wall of the developing cerebral hemisphere, from the pial surface inwards: marginal, cortical plate, subplate, intermediate, subventricular, and ventricular zones (Fig.3).

The pericellular or soluble MMP-2 activity was revealed within the extracellular matrix of the rat visual cortex through the immunohistochemical detection techniques. A brown colored was appeared at the antigen site as a ring or a crescent form in the pericellular matrix of the visual cortex. During prenatal cortical development, the reaction was located within the extracellular matrix of the all developing zones (Figs.4, 5 and 6). While no activity or reaction of TIMP-2 could be depicted through out the extracellular matrix of the zones of prenatal rat visual cortex at any embryonic age.
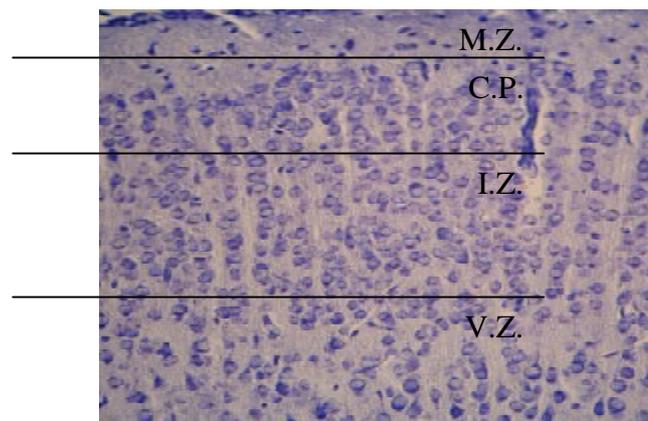
Figure-2: Coronal section through the primary visual cortex at E18. MZ-marginal zone; CP-cortical plate; IZ-intermediate zone; VZ-ventricular zone. Nissl stain (×125).
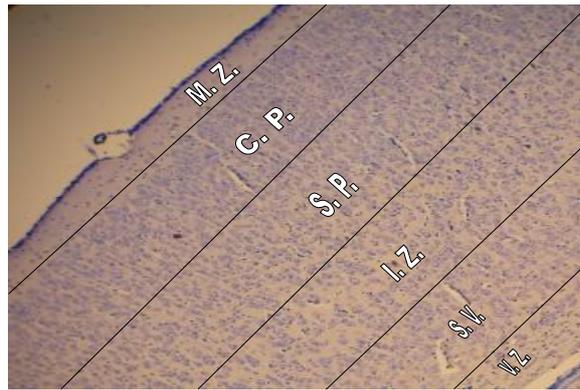
Figure-3: Coronal section through the primary visual cortex at E20. MZ-marginal zone; CP-cortical plate; SP-subplate zone; IZ-intermediate zone; SV-subventricular zone; VZ-ventricular zone. Nissl stain (×100).

Figure-4: Coronal section through the primary visual cortex at E16 stained for MMP-2 with hematoxylin counterstain. Note the pericellular staining of the extracellular matrix (×100).
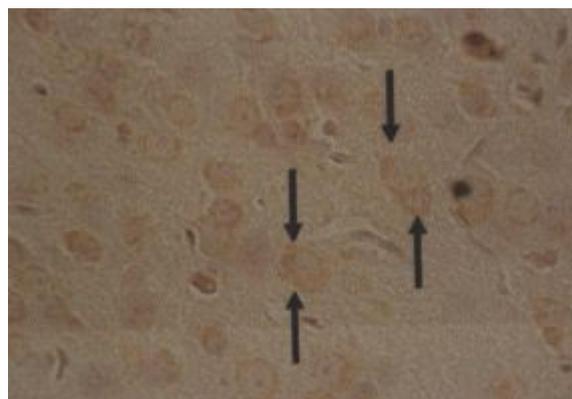
Figure-5: Coronal section through the primary visual cortex at E18 stained for MMP-2 with hematoxylin counterstain. Note the dividing and migrating cells surrounded by ring form pericellular positive matrix (arrows) (×125).

Figure-6: **Coronal section through the primary visual cortex at E20 stained for MMP-2 with hematoxylin counterstain. Note the pericellular staining of the extracellular matrix (×125).**

Discussion:

Studies over the past few years have started to unite the mechanism that support and direct the migration of newborn neurons in which the molecular mechanisms underlying the prenatal development of the central nervous system are largely unknown [8] with the fact that the parameters of the neurons and glial cells change during their developmental course and this affects the animal behaviors [9].

The position of the primary visual cortex region and layers was determined by Nissl staining. The present results confirm and extend previous observations in rat brain. It is clear from the aim of this study that this work was mainly intended to deal with some molecular determinants of ontogeny.

The development of the nervous system requires complex series of cellular programming and intercellular communication events that lead from the early neural induction to the formation of a highly structured central and peripheral nervous system and the role of immunohistochemistry in the study of this development is evident through an increasing number of studies that provide the importance for the role of immunohistochemical markers in nervous system development [2].

In this study, MMP-2 was present in all over the laminae of the developing prenatal visual cortex. MMP-2 can dissolve the chondroitin-sulfate proteo-glycans (CSPGs) during neurite outgrowth and it is noteworthy that MMP-2 localized at the growth cone [10]. Chondroitin-sulfate proteo-glycans are major components of the extracellular matrix of the central nervous system.

During development, CSPGs condense at high concentration in lattice-like structures, designated perineuronal nets which completely ensheath visual cortical neurons [11], this was explained the results of this study in that the MMP-2 expression was in the form of pericellular rings to degrade the CSPGs after its activation in the extracellular space and particularly in the perineuronal cell

body space. CSPGs, which produced by astrocytes, are inhibitory for axonal sprouting and cellular migration [12].

In the brain, MMPs may be involved in a variety of cellular functions depending on the developmental stage of the involved cells [13]; Hehr *et al.* [14] and Mannello *et al.* [15] proved that MMPs are involved in various important cellular functions during development.

The high level of MMP-2 in the prenatal period was important to make extracellular substances as fluid-like as possible, to perform the neurons their migration in a timetable that designed for neuronal migration. So the migration waves of the neurons were traveling in a MMP saturated extracellular matrix.

From this, it can be conclude that MMP-2 expression/activity can be considered as a marker of undifferentiated visual cortex. This might add to the notion propagated by Kaczmarek *et al.* [16] about MMP-2 expression during developmental process, in which, the expression of MMP-2 was increasing during the developmental process, while the expression of the enzyme was decreasing after cessation of the developmental process.

In addition to MMP-2, also TIMPs are found in the extracellular matrix of the brain [16] and the cellular source of TIMP-2 is the neurons [17] and it is secreted in a soluble form into extracellular space [18].

The balance between MMPs and their inhibitors (TIMPs) in the pericellular environment determines the most significant proteolytic events in tissue remodeling [19].

During the prenatal development of the visual cortex, as has been noticed in this study, the apparently homogenous cells will differentiated into the six zones of the prenatal visual cortex, in which cell-cell and cell-matrix interactions play critical roles in all phases of developmental tissue remodeling.

In this study, the changing pattern of the markers reflected sequential growth related modification of the cell components toward the establishment of an adult type.

Prenatally, TIMP-2 has no expression/activity in the developing visual cortex; this finding was in agreement with that of Tong et al. [20]. Tissue inhibitor of metalloproteinase-2 (TIMP-2) reduces extracellular matrix proteolysis [21], hence it can inhibit cell migration [22], and thus it has no role during this period of development [23]. The appearance of the activity of TIMP-2 in the brain at early postnatal period means cessation of cellular migration with beginning and maintaining of the neuronal differentiation postnatally [17].

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