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In Situ Hybridization probing by Transforming Growth Factor β1 marker during prenatal period for rat's primary visual cortex

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الخلاصة:

الهدف من الدراسة هو لربط التغيرات التطورية ماقبل الولاده للقشرة البصرية الاولية مع الظهور المكاني والزماني لفعالية المسبار المدروس من خلال فحص التهجين النسجي. المؤشر TGF−β1 له وظائف متعددة، متضمناً كلاً من تشجيع تشكيل وايضاً تثبيط أنحلال الانسجة الرابطة مع اعاقة تزايد الخلايا الظهاريّة.

تم استخدام (18) ثمانية عشر من أجنة الجرذ وبواقع (3) ثلاثة اجنة لكل عمر جنيني (يوم 15، يوم 16، يوم 17، يوم 18، يوم 19، ويوم 20). إن ظهور فعالية المسبار الجزيئي تكون تدريجية وتبتدأ من الطبقات العميقه باتجاه الطبقات السطحية للقشرة البصرية الاولية للجرذ.

هجرة الخلايا ستتغير أعتماداً على طريقة ظهور تراكيز المسبار الجزيئي او المؤشر TGF−β1: ففي التراكيز المنخفضة للمسبار، هجرة الخلايا تكون سهله بينما في التراكيز العالية للمسبار فإن هجرة الخلايا تكون صعبة، لذا تظهر أهمية المسبار الجزيئي في تنظيم سلوك الخلية خلال الحياة ما قبل الولادة.

Abstract:

The objective of this study is to recount the prenatal developmental changes of the primary visual cortex with the space and time appearance of the activity of the studied marker through In Situ Hybridization technique. TGF-β1 has a multitude of functions, including both promoting the formation and inhibiting the breakdown of connective tissue with inhibition of epithelial cell proliferation.

Eighteen embryos were used; three embryos for each of the following gestational days (G15, G16, G17, G18, G19 and G20). The reaction was gradual and followed an inside-out gradient from deep to superficial laminae of the rat's primary visual cortex.

Cell migration was altered by TGF-β1 in a concentration-dependent manner: at low concentrations, cell migration was promoted whereas at high concentrations, migration was impeded. Therefore, it is an important regulator of the cell behavior throughout prenatal age.

Introduction:

There are two classes of polypeptide growth factors, TGFα and TGFβ, which are not related to one another and are acting through different receptor mechanism.

There are presently twenty four known members of TGFβ subfamily in mammals. TGF-β1 is belonging to Bone Morphogenetic Proteins (BMPs) family that has a critical regulator of morphogenesis and axial specification. These factors were first identified on the basis of their effect on bone formation [1].

The most pronounced differences in the TGF-β isoforms are their spatially and temporally distinct expression of both the mRNAs and proteins in developing tissues in which different isoforms of TGF-β are encoded by different genes [2]. This family of growth factors employs a unique receptor complex initiating the intracellular signaling events [3].

Glial cells have been identified as growth factor providers, some growth factors decrease while others increase during the brain developments, this regulation of growth factors expression may facilitate the survival and function of the neurons [4]. They generally influence the development or survival of multiple cell types, including neurons, astrocytes and oligodendrocytes through the presence of appropriate receptors [5]. However, the TGF-β related factors have important roles in the nervous system, where they are thought to act principally through autocrine and paracrine mechanisms. They are multifunctional peptide that controls proliferation and differentiation [3], acting through membrane receptors and plays a central role in the regulation of cell growth and differentiation with either stimulatory or inhibitory effects, depending on the background of its action. They can also interact with other growth factors to stimulate or inhibit their action. Growth factors of the TGF-β class play important roles in neural development [6]. TGF-β1 is the prevalent form and is found almost in all places and the mechanisms of its activation are not known in detail[2].

TGF-β1 regulates cell migration and it is acting through membrane receptors to signal proliferation and differentiation in many different cell types. Cell migration was altered by TGF-β1 in a concentration-dependent manner: at low concentrations, cell migration was promoted whereas at high concentrations TGF-β1 impeded migration[7]. TGF-β1 is expressed principally by glial cells and by neurons; it is not present in significant amounts in the mature nervous system. It has anti-inflammatory effects and has an important regulator of immune and inflammatory processes in the central nervous system [8].

TGF-β1 is thought to function principally following injury to the nervous system, where its expression is dramatically induced by microglial cells. In some neurons, TGF-β1 is a component of the response to neurodegeneration or trauma and its synthesis and secretion are elevated in these settings. The synthesis of TGF-

β1 following injury in the nervous system is consistent with its effects as inflammatory responses in other organ systems. TGF-β1 plays an instructive role in specifying cellular phenotype [9].

TGF-β1 affects cell migration and can promote the migration of a variety of non-neural cells. The studies provide compelling evidence that the TGF-β system is involved in neuronal migration in the developing brain. TGF-β1 can influence cell migration by modulating the expression of the integrins (as cell adhesion proteins) that mediate attachment and promote motility [2].

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 [10].

In over all organization the telencephalon could be divided into a series of layers, named according to Boulder Committee [11], from the pial surface inwards as the following zones: marginal, cortical plate, subplate, intermediate, subventricular, and ventricular. 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.

This prenatal developmental study is done by probing the primary visual cortex, in rat's embryo from prenatal (embryonic) day fifteen (E15) till birth (E21) using the TGF-β1 In Situ Hybridization marker or technique for formalin-fixed and paraffin-embedded tissue.

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

Material 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, three embryos for each of the following gestational days (G15, G16, G17, G18, G19 and G20) were involved in this study. To include an embryo under a particular embryonic age, 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.

In Situ Hybridization method involves deproteinization of fixed tissue sections mounted on slides, hybridization of the target nucleic acid sequences with a DNA or RNA probe, and detection of the hybridized probe to permit microscopic examination. The most widely used non-radioactive technique entails labeling the probe with biotin. The hybridized probe is then detected by addition of enzyme-conjugated streptavidin followed by a suitable enzyme substrate, which produces a colored end product visible, by light microscopy [12]. This conventional colorimetric reaction avoids the health hazards, disposal problems, and inherent instability of radiolabeled probes.

Serial sections of (5µ) thickness were obtained in the coronal plane for In Situ Hybridization staining technique with DNA Probe Hybridization/Detection System - In Situ Kit from (Maximbio). DNA Probe: Biotinylated long DNA for rat TGF- β1. Cat. No.: IH- 60082. Size: 250bp. This biotin-conjugated probe was produced by Polymerase Chin Reaction (PCR) primers (Maxim's product Catalog # SP-10610) under PCR conditions using Rat cDNA and biotin-dUTPs & biotin-dATPs. Purity: the biotin-conjugated probe was purified by cartridge and showed a single band on gel. Sequences: (Alignment on database: Genbank, NM 021578). Form: Liquid 8 µg biotin-labeled DNA in 100 µl deionized distilled water ddH2O).

Since the visual cortex cannot be recognized easily in 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 [13]. So in embryonic stages the caudal part of the developing neopallium was chosen to represent the visual cortex in this study.

Results:

The major hitch of In Situ Hybridization technique is to save its reaction without sacrificing the quality of cell morphology [14]. However, at G15, the reaction was observed in whole zones or laminae of the prenatal developing primary visual cortex (Fig. 1).

At G16, the reaction was observed in deep zones of the developing primary visual cortex (Fig. 2 and Fig. 3).

At G17, the reaction was observed in the subventricular zone of the prenatal primary visual cortex (Fig. 4).

At G18, the reaction was observed in the cortical plate of the prenatal developing primary visual cortex (Fig. 5).

At G18, the reaction was observed in the cortical plate of the prenatal developing primary visual cortex (Fig. 5).

At G19, the reaction was observed in the cortical plate of the prenatal developing primary visual cortex (Fig. 6), but it was lighter reaction than that of G18.

At G20, no reaction could be depicted through out the zones of the prenatal developing primary visual cortex (Fig. 7).

The superficial laminae or zones are the marginal, cortical plate and subplate. While the deep zones are the intermediate, subventricular and ventricular.

	Visual cortex	G15	G16	G17	G18	G19	G20
TGF-β1	**Deep zones**	++	++	+ +			No reaction
	Superficial zones	++			+ +	+	No reaction

+ +: **dark stain**. +: **light stain**.

Thus, the reaction was gradual and followed an inside-out gradient from deep to superficial laminae, so there were dynamic changes of the appearance of the marker TGF-β1 in the prenatal developing visual cortex.

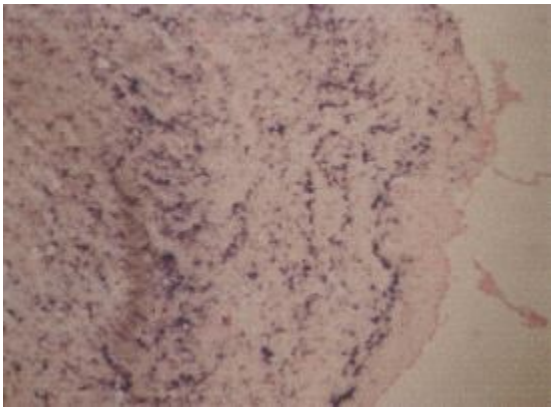

Figure-1: Coronal section through the developing primary visual cortex at G15 stained for TGF-β1 mRNA with nuclear fast red counterstain. Note the staining of the whole laminae (×100).

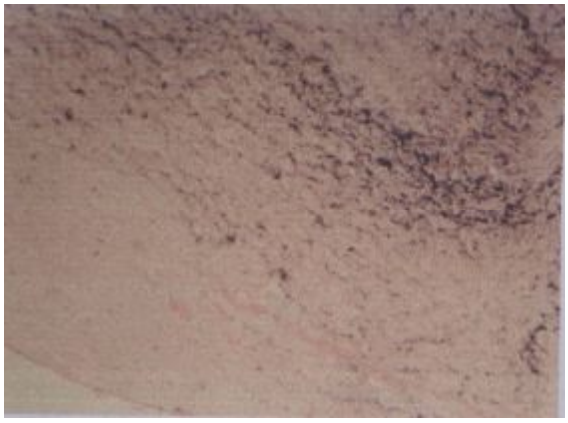

Figure-2: Coronal section through the developing primary visual cortex at G16 stained for TGF-β1 mRNA with nuclear fast red counterstain. Note the staining of the deep zones (×100).

Figure-3: Coronal section through the developing primary visual cortex at G16 stained for TGF-β1 mRNA with nuclear fast red counterstain. Note the staining of the deep zones (×100).

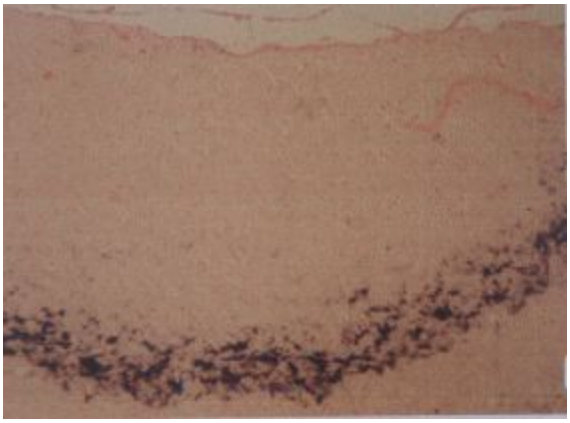

Figure-4: Coronal section through the developing primary visual cortex at G17 stained for TGF-β1 mRNA with nuclear fast red counterstain (×100).

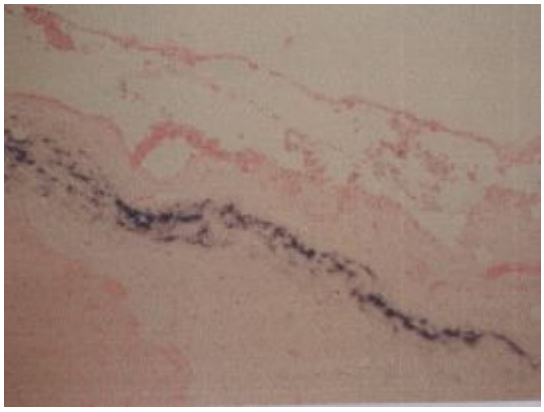

Figure-5: Coronal section through the developing primary visual cortex at G18 stained for TGF-β1 mRNA with nuclear fast red counterstain (×100).

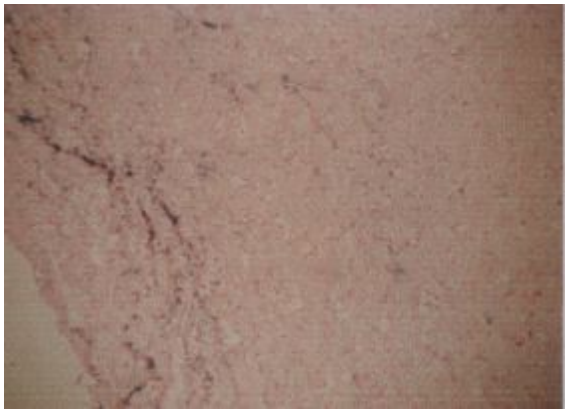

Figure-6: Coronal section through the developing primary visual cortex at G19 stained for TGF-β1 mRNA with nuclear fast red counterstain (×100). Note the lighter stain of the superficial zones in comparison with figure-5.

Figure-7: Coronal section through the developing primary visual cortex at G20 stained for TGF-β1 mRNA with nuclear fast red counterstain (×100).

Discussion:

The In Situ Hybridization technique is used to label TGF-β1 mRNA expressing cells in the rat primary visual cortex using oligonucleotides complementary to its respective mRNA.

TGF-β1 mRNA is expressed in both glial cells and neurons in spatio-temporal pattern to regulate cell proliferation and migration [15]. It is thought to act principally through autocrine and paracrine mechanisms [16].

In this current study, the direction of appearance of TGF-β1 mRNA in prenatal primary visual cortex was followed an inside-to-outside pattern from deep to superficial zones. Receptors were increased during the prenatal period. TGFβ ligands and receptors are strategically placed both in time and space to regulate cell proliferation and migration [9]. TGF-β1 has been reported to play an instructive role in specifying cellular phenotype [16] by mediating appropriate attachment in the extracellular environment and transduce signals to cytoskeletal elements which are important for cell motility, thus it is an important regulator of the cell behavior [17], transforming growth factor-beta (TGF-beta) family members are secreted multifunctional cytokines that play pivotal roles in development and disease. Cell migration was altered by TGF-β1 in a concentration-dependent manner: at low concentrations, cell migration was promoted whereas at high concentrations, migration was impeded [7,18].

During the development of the nervous system, neurons must first migrate to their appropriate locations and then send out axons to make connections. Axonal branches and synaptic contacts are often formed during prenatal development, after axons reach their target regions, neuronal contacts are created through the formation of synapses. Several groups of ligand-receptor pairs have been identified

to regulate each of these cellular events. Evidence also indicates that the same molecules may be used in multiple developmental processes [19]. Throughout the prenatal development, cell proliferation and cell cycle exit are carefully regulated to ensure that the appropriate numbers of cells are produced. Physiologically speaking, a given neuron does not express a growth-factor receptor until it is ready for interaction with its ligand [20]. The antiproliferative agent transforming growth factor-β1 and its receptor are endogenously expressed in proliferative zones of the developing cerebral cortex, thus implicating the growth factor in cell cycle regulation [21].

For the duration of migration, there is a control balance between TGFβ1 and various adhesion molecules which are known to transducer extracellular information into cytoplasm, leading to TGF production from the cells, designated ‘’outside-in signaling’’. Such a bi-directional ‘’cross-talking’’ among adhesion molecules and cytokines is most relevant to developmental process [22]. TGFβ1 alters cell migration through the expression of associated adhesion proteins in developing cortex in a progressive concentration-dependent manner [7].

TGFβ1 is a multifunctional protein that plays a central role in the regulation of cell growth and differentiation with either stimulatory or inhibitory effects, depending on the background of its action. The phase of differentiation characterized by interaction of TGFβ1 with other growth factors to stimulate or inhibit their action and it exhibits a neuromodulatory role [23]. These waves of differentiation indicated that the powerful cytokine (TGFβ1) significantly enhanced neuronal survival and modulate cells phenotype [24] by promoting cell cycle exit and then neurite outgrowth extensions [21].TGFβ1 was played an important role in cellular migration depending on the interpretations of Properzi et al [25] with Ge and Greenspan [17], they proved that TGFβ1 is a potent inducer of extracellular matrix formation, it enhances the formation of the chondroitin sulphate proteoglycans through astrocytes and will promote the deposition of extracellular matrix by stimulating the synthesis of matrix proteins and production of type IV collagen, increase the activity of tissue inhibitor of matrix-degrading metalloproteinase, and inhibit the expression of the enzymes involved in the metabolism of extracellular matrix rather than metalloproteinases.

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