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Does Müller Cell Differentiation Occur Prior to the Emergence of Synapses in Embryonic Turtle Retina?

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Abstract

Müller cells are the main glial cells in the retina, and are related to plexiform layer activity. Recent studies have demonstrated that Müller cells are involved in the synaptic conservation, plasticity, development and metabolism of glutamate. During turtle retinal development, layers, cells and synapses appear at different times. The aim of this research is to study the emergence of Müller cells during embryonic development and their relationship with the synaptogenesis. The authors used retinas from *Trachemys scripta elegans* embryos at stages S14, 18, 20, 23, and 26. Some retinas were processed with immunocytochemistry in order to detect the presence of glutamine synthetase in Müller cells, which was used as a marker of these cells. Other retinas from the same stages were processed for ultrastructural studies. Samples were observed in confocal and transmission electron microscopes, respectively. The present results show that glutamine synthetase expression in Müller cells occurs at S18, before the emergence of the retinal layers and the early synapses.

Keywords

Synaptogenesis; retinal development; glutamine synthetase; ultrastructure; immunochemistry

1. Introduction

In recent years, synaptogenesis research has focused on the role of glial cells in the formation and elimination of synapses [1, 2]. In vertebrate retinas, Müller cells represent the main glial cell type and play an important role in the maintenance of retina homeostasis. Their trophic factors and mediators are active players in the synthesis, release and signaling of the synaptic function [3].

GS (Glutamine synthetase) is an enzyme present in all organisms, from bacteria to humans, which catalyzes the amidation of glutamate to glutamine. In the vertebrate retina, GS is

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found exclusively within Müller cells [4-6], and it plays an important role in controlling the level of extracellular neurotransmitters such as glutamate and GABA [4, 7].

It is thought that GS expression occurs in parallel to the morphological differentiation of Müller cells during retinal development [8, 9] and to astrocyte differentiation in brain development [10-12]. Studies by Prada et al [13] showed that GS expression during development is not related to the degree of differentiation. In fact, early Müller cell differentiation is not detectable by the marker proteins available to date [14]. However, Peterson et al [15] showed that GS is expressed early in development, and they described the process of Müller cell differentiation using a biphasic model in which different markers appear according to the development stage.

During the embryonic retinal development, before light responses begin, a transient circuit gives rise to propagating synchronized waves between neighbor ganglion cells [16]. This spontaneous activity could be the starter of the synaptogenesis and regulate the emergence of distinct connectivity patterns [17]. Propagating retinal waves have been also studied in the turtle retina during development [18]. Müller cells could be also participating in this process [3].

The aim of this research was to study Müller cell differentiation in relation to the emergence of synapses in the inner plexiform layer of embryonic turtle retina. To this end, we examined the expression of glutamine synthetase as a marker of the Müller cells. The synapses in the inner plexiform layer were detected using ultrastruc tural techniques.

The main result of this study has demonstrated that Müller cells were differentiated before synapses occurred in turtle retina during embryonic development. This finding suggests that Müller cells may play a decisive role in synaptic plasticity and the emergence of electrical activity during turtle retinal development.

2. Materials and Methods

Data was obtained from embryonic turtle (*Trachemys scripta elegans*) retinas supplied by Kliebert (Hammond, LA). Turtle developmental stages were selected using criteria established by Yntema [19]. The following stages were used: S14, S18, S20, S23 and S26 (hatching). All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Turtle eggs were kept in a humid incubator at 29 °C. Animals were decapitated and pithed, and the eyes were removed quickly and bisected [20]. Once the retinas had been isolated, they were examined using two types of procedures: ultrastructural and immunostaining studies.

2.1 Immunohistochemistry

After isolation, retinas were immediately submerged overnight in 4% paraformaldehyde, 0.1 % glutaraldehyde in 0.1 M phosphate buffer and included in an acrylic resin, LR-White (London Resin Company Ltd). Some semi thin sections were dyed with toluidine blue, while others were used to perform the immunostaining studies. Subsequently, sections were treated with a saturated solution of sodium-periodated for 20 minutes at room temperature and rinsed in 0.1N HCl and distilled water. Nonspecific binding was blocked by incubation in 5% normal goat serum. Sections were incubated overnight at room temperature in a dark humid chamber in rabbit anti-glutamine synthetase (Sigma-Aldrich; dilution 1:500). The following day, slides were washed in PBS and incubated for two hours in CyTM3-conjugated Affinity Pure Donkey anti-rabbit IgG (Jackson Immuno Research Laboratories; dilution 1:100). Sections were washed for 30 min in PBS and cover-slipped with

Vectashield mounting medium. Sections from each turtle eyecup were used for controlling experiments. Immunolabeled sections were examined by confocal microscopy.

2.2 Ultrastructural Studies

For transmission electronic microscopy, the retinas were fixed by immersion in 1% paraformaldehyde, 1.6% glutaraldehyde, and 0.15 Mm calcium in 0.1 M phosphate buffer (pH 7.4) overnight. Then, retinas were washed in 0.1 M phosphate buffer, postfixed in 2 % osmium tetraoxide, and included in an epoxy resin, Epon 812. Thin sections of 70 nm were contrasted with uranyl acetate and lead citrate and observed in a Zeiss EM10C/CR electron microscope.

3. Results

3.1 Retinal Development

Embryogeny lasts about 60 days in the turtle *Trachemys scripta elegans*, divided into 26 stages [19]. Prior to stage 18 (≈ embryonic day 25), there is no clear differentiation in retinal layers. The synaptogenesis of turtle retina begins at S18 when IPL (the inner plexiform layer) first appears throughout the retina. In this stage, the IPL is visible under light microscopy (Fig. Aa1), although our electronic microscopy results show that the IPL is not completely mature at this stage. It appears as a thin zone with many empty spaces, which are rapidly filled by growing neurites (Fig. Aa3). Therefore, this stage is still immature. OPL (the outer plexiform layer) is also immature at S18, being essentially invisible with light microscopy, in contrast to the IPL (Fig. Aa1). It takes about S20 (≈ embryonic day 30) for the OPL to become visible under light microscopy (Fig. Bb1). However, this layer does not present synaptic contacts. Moreover, at this stage, electron microscopy shows that the empty extracellular spaces are practically absent in the IPL (Fig. Bb3). Synaptic contacts, such as conventional synapses and occasional synaptic ribbons appear for the first time in this layer, but they are scarce. At S23 (≈ embryonic day 40), the OPL and IPL layers are evident (Fig. Cc1). The electronic microphotograph reveals abundant synapses in plexiform layers (Fig. Cc3). Furthermore, in the IPL there are more synaptic contacts, conventional synapses and synaptic ribbons than at S20. At hatching (S26), all layers and synapses in the retina are well defined (Fig. Dd1-d3).

3.2 Development of GS Immunoreactivity

In stages prior to S18, the retina showed negative immunoreactivity to GS. The first positive GS immunoreactivity appears at S18 (Fig. Aa2). At this stage, Müller cells show a low labeling intensity in INL (the inner nuclear layer) and GCL (ganglion cell layer). Five days later, at S20, a large increase in GS expression is seen in the INL and the GCL (Fig. Bb2). This period shows an intensive staining of inner Müller cell processes and the granular pattern of Müller cell bodies and Müller cell endfeet can be observed. At S23, Müller cells show more GS immunoreactivity than at previous stages. This increase is more noticeable in the INL than in the endfeet region of Muller cells (Fig. Cc2). Finally, at S26, the granular pattern of GS changes to a diffuse pattern with a Müller cell shape throughout the retina (Fig. Dd2).

4. Discussion

In order to understand the possible influence of Müller cells in retinal synaptogenesis, we studied their appearance during retinal development using GS immunoreactivity in the retina, and this was used as a specific marker for these cells [4, 6]. In fact, GS is the earliest described marker of Müller cells in zebrafish [15] and other species [21]. In our study, GS immunoreactivity was first observed at S18, when the IPL had developed, separating the

retina into a definitive GCL and INL [20]. These findings corroborate the results of previous reports in which labeling of Müller cells occurs before the completion of lamination; the IPL has taken shape while the OPL has not completed development.

The early appearance of Müller cells suggests an important role in neurogenesis [15]. As has been reported in other papers, during development, glial cells function as a scaffold and are involved in axonal migration in nervous tissues [22-28]. Moreover, the observation that at S18, GS immunoreactivity is very low could be because it is basal or constitutive [29]. In effect, if GS is basal at S18, it cannot be involved in the degradative metabolism of extracellular glutamate released by neuronal activity, since there are no differentiated synapses in either of the plexiform layers at this stage [30-32]. In fact, in our results at this stage, the electron microscopy shows an IPL as a thin acellular zone consisting of immature neurites and growth cones scattered within large extracellular spaces and without synaptic contacts. On the other hand, later in development, glial cells promote synapse formation and plasticity [2, 33]. As already mentioned in the results section, retinal synaptogenesis in turtle starts at S20, when plexiform layers have been formed and both conventional synapses and occasional synaptic ribbons appear. From S20 to hatching, the number and maturation of synaptic contacts increase. This increase in synaptic contacts during development occurs in parallel with the increase in GS immunoreactivity from S20 to hatching. These results are consistent with those reported by Prada et al. [13] in rats.

5. Conclusions

GS labeled Müller cells were observed in *Trachemys scripta elegans* retinas from S18, prior to complete emergence of retinal layers and initiation of synapse formation at S20. At S23 (≈ embryonic day 40), the OPL and IPL are evident and electronic microphotographs show abundant conventional and ribbon synapses. GS labeling indicates a progressive increase between S18 and S26, suggesting that Müller cells may play an important role in synaptic plasticity during turtle retinal development.

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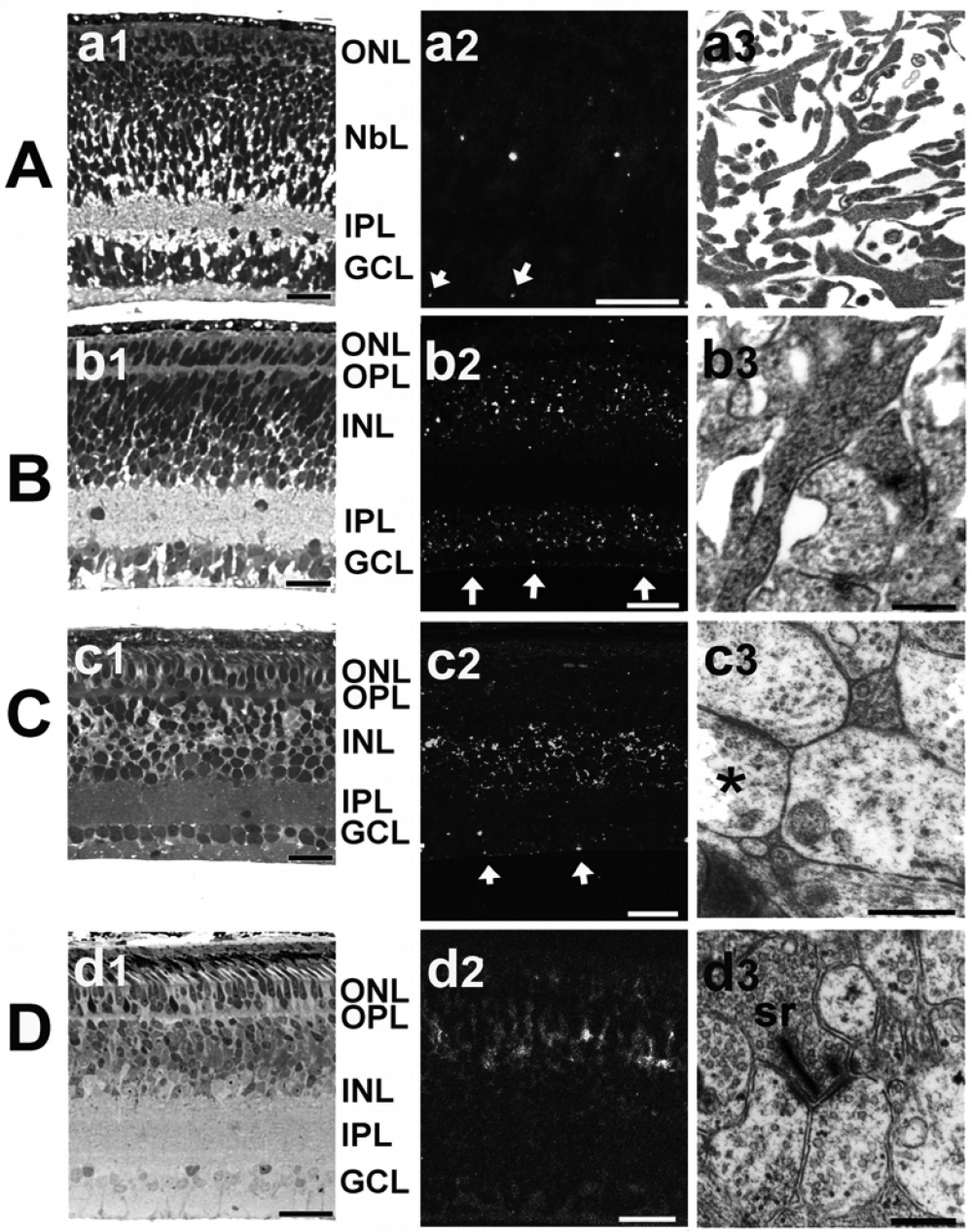

Fig. 1. Photomicrographs from embryonic turtle retinas

Stage 18 (A), S20 (B), S23 (C) and S26 (D). (a1, b1, c1 and d1) Photomicrographs taken from 1 μm-thick vertical semi-thin section stained with toluidine blue. (a2, b2, c2 and d2) Confocal photomicrographs taken from 1 μm-thick vertical semi-thin section processed for GS immunoreactivity. (a3, b3, c3 and d3) Electron micrographs taken from 70 nm-thick vertical thin-sections of IPL. (A) OPL is not clearly visible in contrast to the IPL (a1). NbL and endfeet region of Müller cells (arrows), show a scanty GS labeling (a2). In electron micrographs, IPL appears as an area with empty spaces, filled by growing neurites, without synapses (a3). (B) OPL becomes visible (b1), and a large increase in GS expression is observed in the INL and the GCL (b2). Extracellular spaces and the synaptic contacts are scarce in the IPL (b3). (C) All layers are well identifiable (c1). INL, GCL and endfeet region (arrows) show GS immunoreactivity (c2). IPL show clear synaptic contacts (c3), with

synaptic vesicles (*). (D) All layers are mature and well defined (d1). The pattern of GS labeling is here more diffuse and locate in the middle INL (d2). IPL show clear synaptic contacts (d3), with synaptic vesicles and synaptic ribbons (sr). ONL, outer nuclear layer; NbL, neuroblastic layer, OPL, outer plexiform layer, INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. GS, glutamine synthetase. Scale bars 25 μm (a1-a2; b1-b2; c1-c2; d1-d2); 0.5 μm (a3; b3; c3; d3).