



The Transient Intermediate Plexiform Layer, a Plexiform Layer-like Structure Temporarily Existing in the Inner Nuclear Layer in Developing Rat Retina

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The retina is a highly specialised part of the brain responsible for visual processing. It is well-laminated; three layers containing five different types of neurons are compartmentalised by two synaptic layers. Among the retinal layers, the inner nuclear layer (INL) is composed of horizontal, bipolar, and amacrine cell types. Bipolar cells form one sublayer in the distal half of the IPL, while amacrine cells form another sublayer in the proximal half, without any border-like structure. Here, we report that a plexiform layer-like structure exists temporarily in the border between the bipolar and amacrine sublayers in the INL in the rat retina during retinal development. This transient intermediate plexiform layer (TIPL) appeared at postnatal day (PD) 7 and then disappeared around PD 12. Most apoptotic cells in the INL were found near the TIPL. These results suggest that the TIPL may contribute to the formation of sublayers and the cell number limit in the INL.

Key words: Apoptosis, Development, Positioning, Retina

INTRODUCTION

The retina is a part of the central nervous system (CNS) that originates from the neural ectoderm during development. The retina develops from an apparently homogeneous collection of cells in a single-layered neuroepithelium. During maturation, it develops into a laminated tissue with five major neuronal cell classes (photoreceptors, bipolar, horizontal, amacrine, and gan-

glion cells), each of which occupies a characteristic position within the retina. Retinal cell differentiation proceeds in a highly conserved histogenic order. Ganglion cells are generated first, followed in overlapping phases by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells, and finally, Müller cells [1-3]. A number of transcription factors or homeodomain proteins are known to be involved in retinal cell fate determination and differentiation [4, 5]. During this process, two synaptic layers of the retina are formed and retinal cells are accurately positioned. The inner plexiform layer (IPL) appears as ganglion cell dendrites grow, creating a demarcating border between amacrine cells in the inner nuclear layer (INL) and ganglion cells in the ganglion cell layer (GCL). The outer plexiform layer (OPL) is formed when horizontal cell processes are elaborated laterally, generating

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a demarcating border between photoreceptors in the outer nuclear layer (ONL) and bipolar cells in the INL [6]. In this way, the formation of the plexiform layer clearly delineates the position of the main retinal cell classes, although the position of the retinal cell classes is primarily thought to be programmed by intrinsic factors [7]. However, within the INL, bipolar cells are located in the distal half, while amacrine cells are located in the proximal half, without any demarcating border between them.

Previously, Kang and Chung [8] reported development of the substance P (SP) (so called neurokinin 1) receptor-immunoreactive amacrine cells in the rat retina. A distinct plexiform-like structure was found within the INL in developing retina. In this study, we demonstrate that this plexiform layer-like structure, the transient intermediate plexiform layer (TIPL) temporarily exists in the middle of the INL, the border between the bipolar and amacrine sublayers in the INL, in the rat retina from postnatal day (PD) 6 to PD 12, during retinal development and further characterize it.

MATERIALS AND METHODS

Animals

Eight litters of Sprague Dawley rats were used for assessment of retinas in various developmental stages. All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea (2013-0088-06) and were consistent with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue preparation

Eight pregnant rats and their litters of pups (four of each age) at PD 5–14, and 28 were used. Adult rats and their litters were killed by an intraperitoneal injection of 10% chloral hydrate (10 ml/100 g body weight). The eyes were enucleated, and they were then cut along the anterior border of the ora serrata. The posterior segments of the eyes were processed as previously described [9].

Toluidine blue staining

After fixation, retinal pieces taken from the central region of the retina were washed in 0.1 M phosphate buffer (PB), pH 7.4, dehydrated in a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon 812 (Polysciences, Warrington, PA). After curing at 60°C for 2 days, the sections were cut in the vitreal-scleral direction at a thickness of 1 µm, using an ultramicrotome (Reichert-Jung, Nussloch, Germany) and applied to slides coated with 0.5% gelatin. The sections were stained with 1% toluidine blue solution after removal of the epon with alcoholic sodium hy-

droxide for 20 min.

Electron microscopy

For electron microscopic observation, the retinal pieces taken from rat pups at PDs 8 and 10, were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in PB for 2 h at 4°C. The retinas were postfixed in 1% OsO₄ in PB for 1 h, dehydrated in a graded series of alcohol, and embedded in Epon 812. After curing at 60°C for 3 days, ultrathin sections (70–90 nm thick) were collected on one-hole grids coated with Formvar. The sections were stained with uranyl acetate and lead citrate, and examined by electron microscope (Jeol 1200EX, Tokyo, Japan).

Immunohistochemistry

The posterior segments of the eyes were immersed in 4% paraformaldehyde in PB for 30 min. The retinas were carefully dissected from the choroid and placed in the same fixative for 2 h at 4°C. The retinas were then washed through several changes of PB, and transferred to 30% sucrose in PB for 5 h at 4°C. They were frozen in liquid nitrogen thawed, and rinsed in 0.01 M phosphate buffered saline (PBS), pH 7.4. Subsequently, 50-µm-thick vibratome sections were made from the central region of the retina. Before immunostaining, the retinal sections were incubated in 10% normal donkey serum in PBS for 1 h at room temperature to block nonspecific binding activity. The sections were then incubated in a rabbit polyclonal antiserum directed against substance P (SP) receptor (Chemicon, Temecula, CA; dilution 1:1000) for 1 day at 4°C. After washing in PBS for 45 min (3×15 min), the sections were incubated with peroxidase-conjugated goat anti-rabbit-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; dilution 1:100) in PBS for 2 h at room temperature. The sections were then rinsed in three changes of 0.05 M Tris/HCl buffer (TB; pH 7.6) for 5 min each time, at room temperature, and incubated in 0.05% 3,3'-diaminobenzidine tetrahydrochloride in TB for 10 min. Hydrogen peroxide was added to the incubation medium to a final concentration of 0.01%, and the container was shaken gently as the reaction proceeded. The reaction was stopped with several washes of TB or PB after 1–2 min, as determined by the degree of staining. The retinas were mounted using glycerol.

Terminal deoxyribonucleotidyl transferase-mediated d-Uridine 5'-triphosphate Nick-End Labelling (TUNEL) staining and double-labelling

Apoptotic cell death was determined by a modified TUNEL technique according to the manufacturer's instruction (In Situ Cell Detection kit, Boehringer-Mannheim, Mannheim, Germany). After immunostaining for SP receptor, retinal sections were in-

cubated in 0.2% Triton X-100 for 5 min and rinsed in distilled water. Endogenous peroxidase activity was blocked by placing the sections in 3% H₂O₂ in distilled water (DW) for 10 min. The sections were equilibrated in terminal transferase-labelling buffer (Boehringer-Mannheim), and the tailing reaction was performed with terminal deoxynucleotide transferase (0.3 e.u./ μ l) and FITC-conjugated deoxyuridine triphosphate (1 nmol/ μ l) in a humidified chamber for 1 h at 37°C. The reaction was terminated by incubation in a solution containing 0.3M NaCl, 0.03 M sodium citrate. Sections were then incubated at 60°C for 1 h served as positive controls, and staining of negative controls was performed in the absence of terminal transferase.

For double-labelling, the retinal sections processed for TUNEL staining were incubated in PBS containing 10% normal donkey serum after washing in DW and PBS. The sections were then incubated for 1 day at 4°C in mouse monoclonal anti-glutamine synthase (GS), a specific marker for Müller glial cells [10]. After washing in PBS for 45 min, the sections were incubated in appropriate Cy3-conjugated secondary antibodies (Jackson; dilution 1:500), according to the primary antibodies, for 2 h at room temperature. The sections were then rinsed in three changes of PB and cover-slipped.

SP-saporin (SAP) injection

At PD 4, rats were anaesthetised with diethylether, and 5 μ l of sterile isotonic saline or saporin conjugate SP-SAP [11] or free SAP (both from Advanced Targeting Systems, San Diego, CA; 2.6 ng dissolved in sterile isotonic saline) was injected into the vitreous humour of the right eye via a glass micropipette attached to a 10 μ l Hamilton syringe. Rats were sacrificed at PD 14, and retinas were prepared and further processed for immunohistochemistry, as previously described. Following primary and appropriate peroxidase-conjugated secondary antibodies were used: rat polyclonal anti-glycine transporter 1 (from Dr. D. V. Pow, of the University of Queensland, Brisbane, Australia; dilution 1:1000) or rabbit anti- γ -amino butyric acid (GABA; Chemicon; dilution 1:1000) as amacrine cell markers [12, 13] or rabbit anti-recoverin (from Dr. K.-W. Koch, of the Institut für Informationsverarbeitung, Jülich, Germany; dilution 1:20,000) as an ON and an OFF cone bipolar cell marker [14].

RESULTS AND DISCUSSION

In consistent with a previous report [8], a distinct type of cells showing SP receptor immunoreactivity was observed in the developing rat retina (Fig. 1A and B). This type of labelled cell was located in the middle of the INL. They had horizontal processes emerg-

ing from their primary dendrites, and these horizontal processes formed plexuses in the plexiform layer-like structure within the INL. We called this structure the TIPL, because it was temporarily observed during retinal development. The TIPL appeared as a dotted line (Fig. 1C), suggesting that the mature retina is composed of columnar units of cells that are clonally derived [15, 16]. They were only observed at postnatal day PD 6 to PD 12, frequently PD 7 to PD 10, as reported [8].

In order to explore the TIPL, we carefully examined vertical sections of developing rat retinas stained with toluidine blue (Fig. 1D, 2). As shown in Fig. 1D, the TIPL was located at the border be-

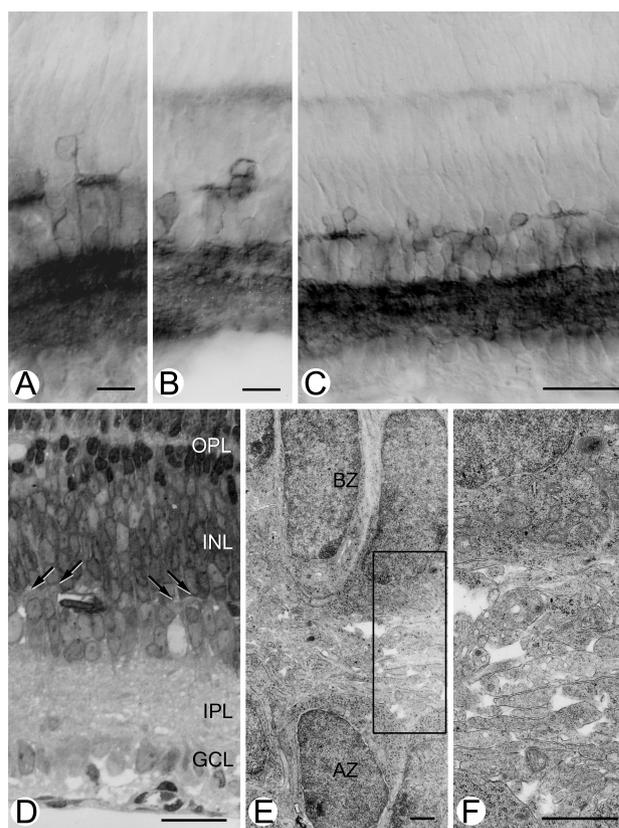


Fig. 1. The transient intermediate plexiform layer (TIPL), a plexiform layer-like structure in the developing rat retina. (A, B) Two examples of a distinct type of cells found in postnatal day (PD) 7 (A) and PD 10 (B) rat retinas immunostained for substance P (SP) receptor. Their processes ramify and form a plexus in the TIPL in the proximal or middle of the inner nuclear layer (INL). OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer. (C) Lower magnification view of PD 7 rat retina immunostained for SP receptor. The TIPL is seen to be a dot line within the INL, not a continuous band. (D) Light micrograph of PD 7 rat retina stained with toluidine blue showing the presence of the TIPL (double arrows) in the INL. (E, F) Electron micrograph shows that the TIPL is located at the border between the bipolar (BZ) and amacrine cell zone (AZ) of the INL (E) and is composed of numerous cell processes in higher magnification view (F). Scale bars=10 μ m in A, B; 25 μ m in C, D; 1 μ m in E, F.

tween the bipolar and amacrine cell zone in PD 7 retinas (arrows), which corresponded to the location at which horizontal processes of one type of SP receptor-immunoreactive cells ramified and formed a plexus (Fig. 1C), and appeared to be simply a patch-shaped extracellular matrix. However, electron microscopic observation clearly demonstrated that this structure was composed of numerous cell processes (Fig. 1E and F), like the IPL and the OPL.

During the retinal development of the mouse and rat, the IPL appears before birth and the OPL is formed at about PD 5. At PD 5, the OPL becomes visible as a thin, pale stained line formed by processes arising from horizontal cells (Fig. 2). The OPL separates the rods into two groups. Rod nuclei situated on the inner side of that layer immediately move across it to enter the ONL, but numerous cells die during retinal migration [17, 18]. Thus, the OPL seems to function as a filter or barrier of migration and thus, struggling inner rods seem to be destined to be broken down. Similarly, the TIPL and a distinct type of SP receptor-immunoreactive cells, of which horizontal processes constitute the TIPL, may play a role in dividing the INL into bipolar and amacrine cell sublayers, by filter-

ing the inappropriately localised bipolar and amacrine cells, or by interrupting their axonal growth onto the IPL and the formation of synapses. This idea may be supported by the finding that the TIPL was observed frequently at PD 7 to PD 10, but not at PD 13 (Fig. 2), similar to the time-course of SP receptor-immunoreactive cells (Fig. 2 in Kang and Chung [8]). The period of the presence of this structure coincides with that of the apoptosis of bipolar and amacrine cells in the INL [19].

To test the above working hypothesis, we performed triple-labeling experiments including TUNEL staining, to determine the relationship between the TIPL and apoptotic bipolar and/or amacrine cells. However, some bipolar cells develop and differentiate late, and thus, their markers are not generally expressed at PD 10 [20]. We indirectly tested this using glutamine synthase (GS), a specific marker for Müller glial cells [10]. As shown in Fig. 3A and B, TUNEL staining revealed that numerous apoptotic cells were present in the middle portion of the INL. In particular, apoptotic cells observed at this time were found near the TIPL (Fig. 3C~E).

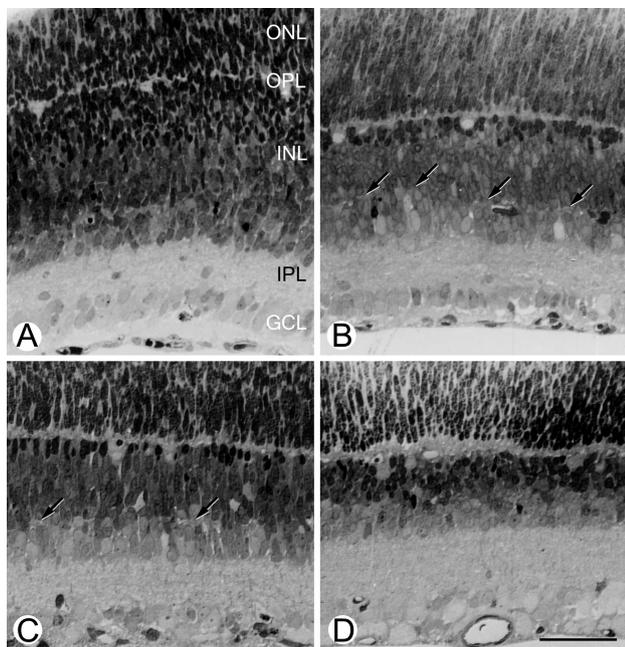


Fig. 2. Advent and disappearance of the transient intermediate plexiform layer (TIPL) during retinal development of the rat. Photomicrographs taken from vertical sections from retinas at postnatal day (PD) 5 (A), PD 7 (B), PD 10 (C), and PD 14 (D) stained with toluidine blue. (A) The outer plexiform layer (OPL) starts to be visible, and thus, five retinal layers are distinguished at this age. (B) Patch-shaped TIPLs (arrows) are frequently found in the middle or proximal part of the inner nuclear layer (INL), between bipolar and amacrine cell groups showing different staining intensity. (C) At this age, the TIPL is still observed at a similar position as shown in (b). However, they are less frequently observed than at PD 7. (D) The TIPL in the INL are no longer found at this age. Scale bar=50 μm.

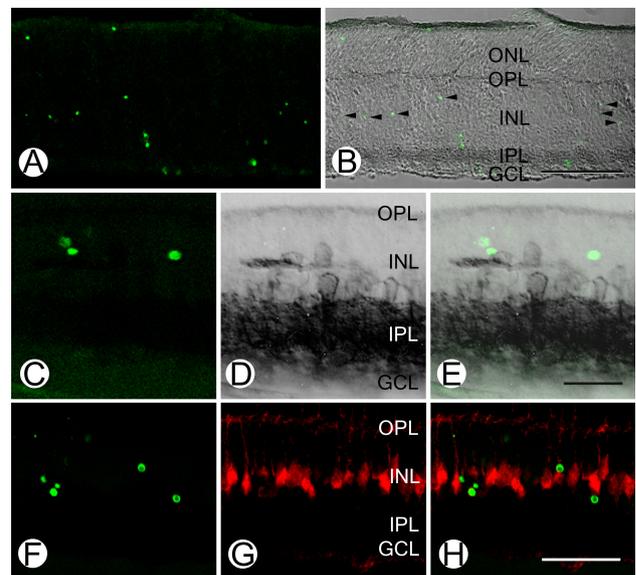


Fig. 3. Relationship between the transient intermediate plexiform layer (TIPL) and apoptotic cells in developing retina. (A, B) Low-power magnification view of postnatal day (PD) 7 rat retina processed for TUNEL staining. Numerous TUNEL-positive cells (green) are found across the retina (A). Among them, more than half are located in the middle or distal portion of the INL in the merged image (B). (C~E) Higher magnification view of PD 9 rat retina processed for TUNEL staining (C) and substance P (SP) receptor immunoreactivity (D). TUNEL-positive cells (green) are frequently found near the TIPL showing SP receptor immunoreactivity in the INL in the merged image (E). (F~H) Confocal images of PD 10 rat retina processed for TUNEL staining (F) and glutamine synthase (GS) immunoreactivity (G). Several TUNEL-positive cells (green) are seen in the INL (F) and numerous GS-immunoreactive Müller glial cells (red) are visible (G). In the merged image (H), colocalization cannot be seen. Scale bar=100 μm in B; 25 μm in E; 50 μm in H.

In addition, the majority of TUNEL-positive cells (96/98) did not show GS immunoreactivity (Fig. 3F~H), suggesting that apoptotic cells may be immature neuronal cells, that is, immature bipolar and/or amacrine cells.

Furthermore, we attempted to ablate TIPL or SP receptor-immunoreactive cells selectively using SP-SAP [11, 21] to demonstrate structural changes of the INL of the retina after the disruption of the TIPL, expecting that bipolar and amacrine cells would be intermingled in the middle of the IPL without distinct sublayers.

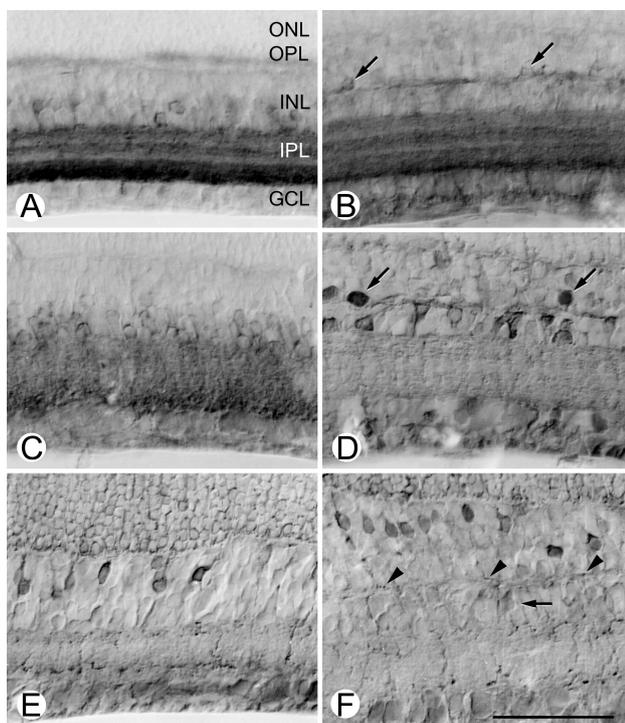


Fig. 4. Changes in retinal organization after injection of substance P-saporin (SP-SAP) into the developing rat retina. Photomicrographs taken from vertical sections from control (A, C, E) and injected (B, D, F) retinas of postnatal day (PD) 14 rats immunostained for SP receptor (A, B), glycine transporter 1 (GLYT-1; C, D), and recoverin (E, F). (A) SP receptor-immunoreactive cells are seen in the proximal part of the inner nuclear layer (INL). (B) The distinct type of SP receptor-immunoreactive cells that are normally found at PD 6 to PD 12, remain at this age and a relatively continuous transient intermediate plexiform layer (TIPL) is also clearly seen. (C) GLYT-1-immunoreactive amacrine cells are seen in the proximal part of the INL. (D) Several GLYT-1-immunoreactive amacrine cells are found in the proximal part of the INL and two ectopically located GLYT-1-immunoreactive amacrine cells (arrows) of which soma are located in the middle part of the INL and processes are within the TIPL, are also found. (E) Recoverin-immunoreactive bipolar cells located in the distal part of the INL and their axon terminals ramified in the IPL are clearly seen. (F) Several recoverin-immunoreactive bipolar cells are found in the distal part of the INL and one ectopic recoverin-immunoreactive bipolar cell (arrow) located in the proximal part of the INL is clearly seen. In addition, some axons of recoverin-immunoreactive bipolar cells (arrowheads) ramify and terminate in the TIPL within the INL. Scale bar=50 μ m.

Unexpectedly, however, SP receptor-immunoreactive cells and the TIPL remained and could be clearly observed apparently at PD 14. Moreover, processes of some amacrine cells and axons of some recoverin-immunoreactive bipolar cells ramified and terminated in the TIPL (Fig. 4). These results could be interpreted as demonstrating plasticity in development due to the application of toxin to the developing retina.

A previous report by Johansson and Ehinger [22] was related to these unexpected results described above. In their study, the TIPL, or a similar structure, was observed in the middle of the INL in explanted neonatal rat retina after 11 days *in vitro*. They described it as an ectopic neuropil area. In addition, they found immunoreactivity to synaptophysin (a presynaptic marker) in the area. Taken together, these findings indicate that the TIPL exists in the INL during retinal development.

In summary, we show that the TIPL, constituted of a morphologically distinct type of cells, including an SP receptor-immunoreactive cell type, temporarily exists in the INL during retinal development, and they appear to be associated with neuronal cell removal *via* apoptosis in the INL. Our results suggest that they contribute to dividing the INL into bipolar and amacrine cell sublayers. Further investigations will be needed to determine whether the TIPL is a general structure appearing during development of all retinas, including human, and to elucidate the role of the cell type that form this structure.

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