

Neuronal Intermediate Filament in the Developing Rat Retina

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The distribution of peripherin mRNA in adult rat retinal ganglion cells revealed by *in situ* hybridization was relevant to the distribution of immunoreactivity to anti-peripherin. Peripherin positive ganglion cells began to differentiate from the 13th gestational day. These peripherin positive ganglion cells expressed α -internexin from the early phase of differentiation and majority of the peripherin positive ganglion cells completed their migration by the 17th gestational day. The optic nerve layer was gradually enlarged during the 15–17th gestational

day. However, unlike adult retina, neurofilament triplets were not revealed in the prenatal optic nerve. A different group of neurons with α -internexin without peripherin increased during the 15–19th gestational day and neurons of the internal nuclear layer seemed to transiently express α -internexin because they were seen in the internal nuclear layer above horizontal cells on the 19th gestational day. Although horizontal cells were clearly identified in the retina on the 19th gestational day, calbindin 28KD was not revealed in this stage.

Key words: rat retina, peripherin, immunohistochemistry, *in situ* hybridization

I. Introduction

The retina differs from all other central nervous tissue by having primary photoreceptor cells. Photoreceptive spots are seen in very low classes of invertebrates such as jellyfish, in which a neural tube, the anlagen of central nervous tissue of vertebrates, is not yet formed [13, 24, 26]. The fundamentals of the photoreceptor system (including biochemical cascades) in various classes of invertebrates have aspects in common with those of vertebrates [5, 20, 21], though the neurons intervening between the photoreceptor cells and ganglion cells are not so complex [13]. Furthermore, highly integrated eye structures, including the lens, iris and retina, are not specific to vertebrates, and are seen in some invertebrates such as mollusca, though the complex inner nuclear layer is not so well developed in them [13, 26]. These findings suggest that the evolution of the photoreceptive system including the whole eye structure is not totally dependent on the evolution of the neural tube. The signal transduction system including rodopsin and related unique proteins such as peripherin Rds [10] is distinct from other central nervous

tissue and seems to reflect early segregation of the photoreceptive system from other neurosensory systems during evolution. α -internexin has been demonstrated in the outer plexiform layer and optic nerve fiber layer of mature retina [6] and immunoreactivity to peripherin, type III intermediate filament [23], has been reported once using a polyclonal antibody [8]. However, peripherin mRNA in the retina has yet to be examined. In this paper, we described the results of our analysis of peripherin mRNA expression in rat retina in comparison with the distribution of peripherin immunoreactivity. In addition, the expression of peripherin and α -internexin in developing rat retina was examined by immunohistochemical staining and we discussed the characteristics of neurogenesis in the retina.

II. Materials and Methods

Materials

Eight to fifteen weeks old male Wister and ACI black rats (both from Clea Japan) were sacrificed under anesthesia with diethyl ether. Eyes and optic nerves were then extirpated from the sacrificed animals. Corneas were removed from extirpated eyeballs with scissors to remove the lens and vitreous humor. The remaining eye tissue including the retina, choroid plexus and sclera and optic nerves were

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rinsed in 0.1 M cold phosphate saline buffer (pH 7.4). For the control of immunohistochemical staining and *in situ* hybridization, transverse sections of adult rat duodenal walls were used.

Immunohistochemistry

Some of these tissue fragments were frozen in an Optimal Cutting Temperature Compound (Sakura Finetechnical Co. Ltd., Tokyo) and sliced into 4 μ m sections for immunohistochemical analysis. The sections were fixed in acetone for 30 to 60 sec and processed for three-step immunoperoxidase staining using DAKO LSAB system, according to the manufacturer's instructions and visualized by 3-3' diaminobenzidine. Primary antibodies used for analysis were monoclonal antibody to peripherin (Novocastra Co. Ltd., Newcastle, dilution: $\times 200$) and rabbit polyclonal antibody recognizing epitope common to human and rabbit α -internexin (Chemicon Co. Ltd.) (dilution: $\times 200$), mouse monoclonal antibody to specific to rat α -internexin (Chemicon Co. Ltd.) (dilution: $\times 500$), monoclonal antibody to neurofilament (2F11, DAKO Co. Ltd). These primary antibodies were applied to frozen sections for 2 hr at 4°C after pretreatment with 0.3% H₂O₂ for 10 min to eliminate internal peroxidase activity. Some of the extirpated tissues were fixed in 20% buffered formalin (pH 7.6) embedded in paraffin and were also used for immunohistochemical analysis. Instead of microwave treatment, deparaffinized 4 μ m sections were autoclaved at 120°C for 1 min in 10 mM citrate buffer (pH 6.0) or in 1 mM EDTA (pH 8.0) before the application of primary antibodies for 18 hr at 4°C [15]. Then, peroxidase labeling was done using a streptavidine biotin system, and the peroxidase product was visualized with 3-3' diaminobenzidine and observed at temporal side, 2–3 o'clock from papilla of optic nerve in comparison with the immunoreactivity of control rat duodenal nerve plexus.

In situ hybridization

Preparation of rat peripherin RNA probe for in situ hybridization

Total RNA was extracted from rat spinal dorsal root ganglia and cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Then, peripherin cDNA was amplified by polymerase chain reaction (PCR). The PCR primers are as follows; sense 5'TCCTGCCAAGCGCTAGAAT-3' and antisense 5'-CGGCCATGGAGAAGTCGA-3'. Amplified cDNA was cloned into pCR II-TOPO vector (Invitrogen, San Diego, CA). Then, digoxigenin-labeled sense and antisense RNA probes were synthesized by *in vitro* transcription using T7 and SP6 polymerase (Roche Diagnostics, Mannheim, Germany), respectively.

Hybridization

For *in situ* hybridization, deparaffinized 4 μ m sections of retina were treated in 1% pepsin (0.1 N HCl) for 6 min at 37°C. Then the sections were dehydrated in ethanol and incubated with RNA probe of rat peripherin conjugated

with digoxigenin (100 μ g/ml) for 24 hr and rinsed in 4Xs standard sodium citrate buffer at 37°C. Mouse monoclonal anti-digoxigenin antibody (0.2 μ g/ml) (Roche Diagnostics, Mannheim, Germany) was applied for 60 min, followed by immunoperoxidase labeling using the streptavidine biotin system. Coloration of peroxidase reaction was detected by 3-3' diaminobenzidine.

III. Results

Mature rats

Neurofilament was demonstrated in the optic nerve layer and in outer plexiform layer both in frozen and paraffin embedded specimens of the Wister and ACI black rats. In the retina, peripherin immunoreactivity was confined to the optic nerve fiber layer (Fig. 1a). The peripherin positive nerve fibers extended into the brain through the optic nerve bundles, and were seen in the perikarya of a few small cells in the inner granular layers. Peripherin mRNA was revealed in most of the ganglion cells and a few of the cells in the inner granular layer. These findings were relevant to the immunohistochemical findings (Fig. 1b).

α -internexin immunoreactivity was detected in the outer plexiform layer, just beneath the inner nuclear layer, and running horizontally (Fig. 1c). Compared to the outer plexiform layer or control tissue, the immunoreactivity of the optic nerve fiber layer was quite weak in frozen sections or in paraffin sections heated in 10 mM citrate buffer (pH 6.0), and was clearly demonstrated only after heat antigen retrieval in 1 mM EDTA (pH 8.0) (Fig. 1c).

Embryonic rats

Neuronal intermediate filament began to be expressed in the retina from embryonic day 13 (E13). At this stage, quite a few of the α -internexin positive neurons and nerve fibers were revealed in the retina, and their distribution was relevant to that of α -internexin. However, neurofilaments had yet to be expressed.

On embryonic day 15, neurons with peripherin and α -internexin increased considerably, especially in the upper half of the retina and optic nerve fiber bundles, with these two intermediate filaments seen at the inner surface of the retina (Fig. 2a, b). Neurofilament was revealed in the peripheral neurons, but not in the retina (Fig. 2c).

On the embryonic day 17, nuclei beneath the optic nerve layer was segregated into an inner layer with enlarged nuclei and an outer layer with smaller nuclei. Neurons with peripherin and α -internexin ceased increasing numerically and almost completely finished migrating to the superficial portion of the upper layer, the final location of ganglion cells, though the inner plexiform layer between the ganglion cell layer and inner nuclear layer was not formed yet. A marked increase in the second group of neurons with α -internexin without peripherin was observed in the inner and outer layer, and their neurites were rectangular to the retinal surface (Fig. 3a, b).

On embryonic day 19, the optic nerve layer was en-

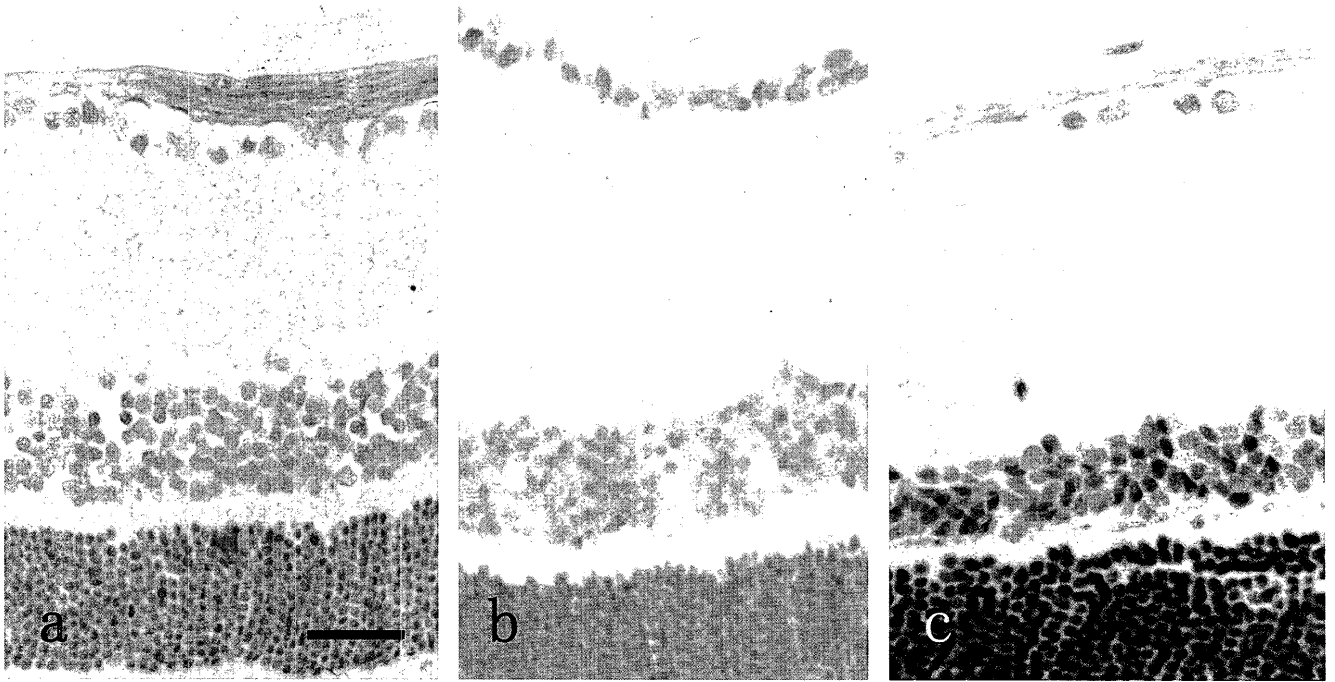


Fig. 1. (a) Peripherin immunoreactivity confined to the optic nerve layer of mature rat retina. (b) Distribution of peripherin mRNA in the mature rat retina. (c) α -internexin in the outer plexiform layer, just beneath the inner nuclear layer and in optic nerve fiber layer of adult rat retina. Bar=50 μ m, counterstain hematoxylin.

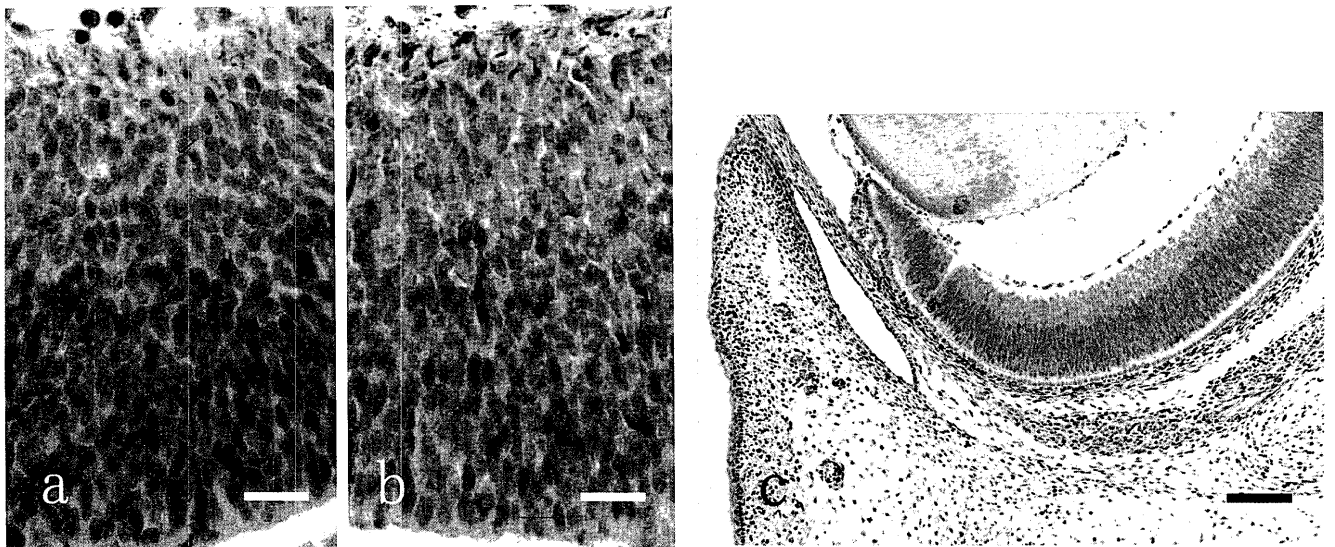


Fig. 2. Distributions of immunoreactivity to peripherin (a) and α -internexin (b) resemble each other in the retina of 15th day embryo. (c) Neurofilament was not expressed in this stage. Bars=25 μ m (a, b); 125 μ m (c), counterstain hematoxylin.

larged, but the distributions of α -internexin positive neurons with or without peripherin were fundamentally the same as the 17th day. A single layer of α -internexin positive and peripherin negative neurons at the junction of two nuclear layers began to extend their nerve fibers parallel to the retinal surface and their appearance was compatible with that of the horizontal cells (Fig. 4). However, calbindin 28KD was not disclosed in these neurons yet. α -internexin positive

cells in the retina were far more abundant than the number of horizontal cells during this period.

IV. Discussion

Originally, peripherin, a type III neuronal intermediate filament distinct from type IV neurofilament, had been identified as an intermediate filament of peripheral neurons [23],

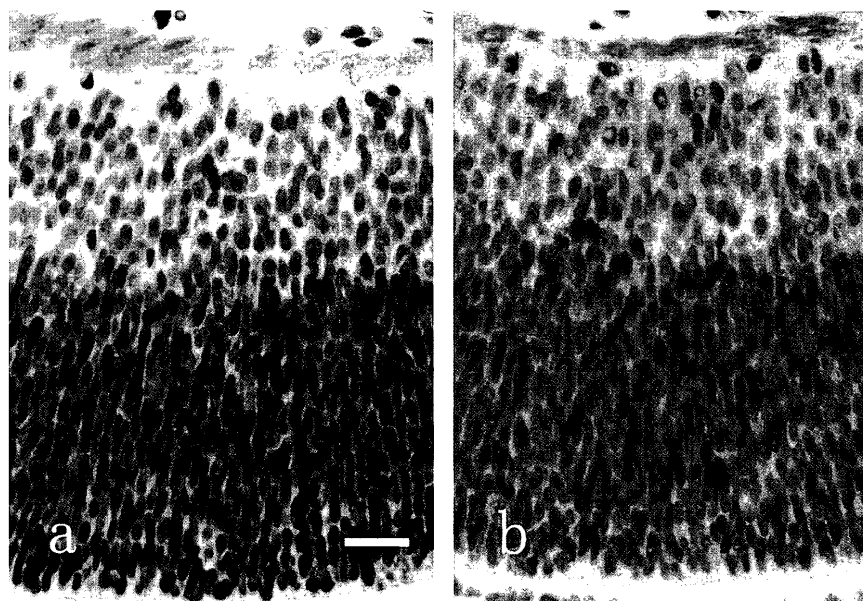


Fig. 3. (a) Peripherin in the optic nerve layer and subjacent neurons. (b) Immunoreactivity to α -internexin distributed in the neurons of the deeper portion of the retina in addition to optic nerves and neurons positive to peripherin. Embryos of 17th gestational day, Bar=25 μ m, counterstain hematoxylin.

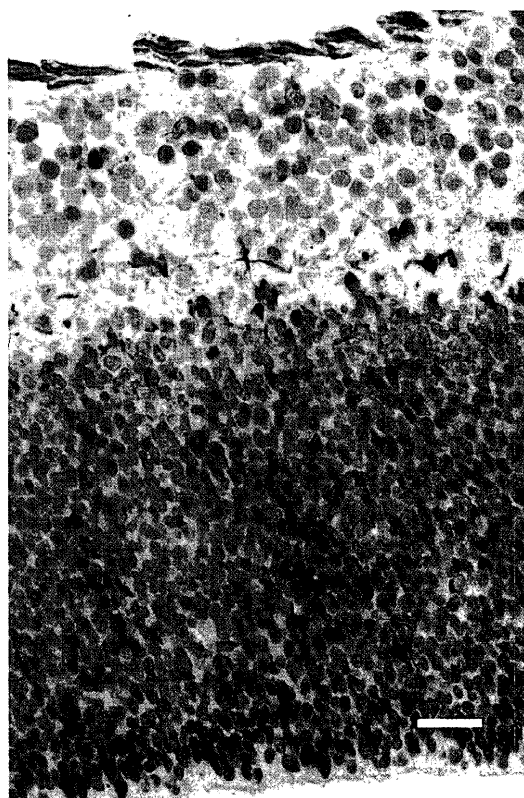


Fig. 4. α -internexin positive lateral sprouting processes of horizontal cells were easily identified. Embryos of 19th gestational day, Bar=20 μ m, counterstain hematoxylin.

but later, this intermediate filament was revealed in the central nervous tissue during the developmental stage and

in some central neurons derived from the neural tube [4, 8]. Our present immunohistochemical and *in situ* hybridization analyses confirmed the expression of peripherin in addition to neurofilament in the ganglion cells [2, 18].

Ganglion cells (primary sensory neurons) of the retina are the fundamental elements of the photoreceptor system seen even in lower classes of invertebrates such as coelenterata [27] and are quite old in their phylogeny [26, 27]. Biochemical cascade involved in the photoreceptor cells is also common among these animals [5, 20, 21]. On the other hand, complex networks of interneurons between the photoreceptor cells and ganglion cells are well differentiated only in higher classes of vertebrates in which retinal anlage is incorporated into the neural tube [13].

Two distinct sequences in the retinal neurogenesis seem to reflect the unique phylogenetic sequences of retinal evolution. Phylogenically older ganglion cells differentiate earlier than the newer interneurons and are characterized to have peripherin and α -internexin from the early phase of differentiation. Late developing α -internexin positive and peripherin negative neurons seem to belong to interneurons such as amacrine cells as in embryonic mice [6]. Horizontal cells are also included in this group and their characteristic lateral sprouting processes with α -internexin were clearly identified on the 19th gestational day though calbindin 28KD was not disclosed yet as reported previously [19]. In the adult retina, immunoreactivity to α -internexin was clearly shown in the outer plexiform layer in frozen sections or paraffin sections heated in 10 mM citrate buffer. However, immunoreactivity in optic nerve fiber layer was decreased and autoclaving in 1 mM EDTA (pH 8.0) was required to demonstrate obvious immunostaining. Similar phenomena have been reported in other CNS neurons and seem to be in-

duced by the proportional decrease of α -internexin content in the optic nerve intermediate filaments [12]. Distribution of immunoreactivity to α -internexin in the outer plexiform layer seems to correspond to horizontal cell processes as reported previously [6]. However, this intermediate filament was not detected in other layers of adult retina even after antigen retrieval and suggested its transient expression in the neurons of inner nuclear layer such as amacrine cells [6].

Various factors are regulating development, maturation and degenerative processes of retinal neurons [14, 17, 22, 28]. Müller cells are shown to have certain trophic effects [30]. In addition, they seem to be involved in the neuronal degeneration mediated by glutamate [3] because they are supposed to have an important role on the glutamate metabolism [29].

Expression of peripherin is not so common in central neurons except for a few examples such as spinal motor neurons. Peripherin is upregulated by NGF [14] or nerve injury [16]. This phenomenon is observed in the spinal motor neurons affected by various motor neuron diseases including ALS [11, 16, 25] and over-expression of peripherin causes late onset of motor neuronal death. This phenomenon is related to p75, a low affinity NGF receptor [1], and a similar phenomenon was reported in the ganglion cells of chick retina [9]. In the optic system, however, such phenomena were not demonstrated in our preliminary study using rat retina as reported previously [7], and the correlation between peripherin expression and re- and degenerative processes of ganglion cells remains to be resolved.

V. References

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