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Original Article

Remodeling processes during neural retinal regeneration in adult urodeles: an immunohistochemical survey

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ABSTRACT Dynamic features of neural retina regeneration in the adult newt *Pleurodeles waltl* were analyzed using immunohistochemical studies. Antibody to Glial Fibrillary Acidic Protein (GFAP) was used as a marker of the retinal glial supportive system in order to obtain an overview of the retinal reorganization pattern. Unexpectedly, retinal progenitor cells displayed GFAP staining, as did later Müller glial processes and astrocytes supporting ganglional axons. To study changes of plasticity during retinal restoration, the expression patterns of highly- (PSA) and weakly-sialylated N-CAM were examined by double staining. In the retina of adult newts, a sustained expression of total-N-CAM and PSA-N-CAM was detected. However, while an intense distribution of N-CAM was observed throughout the retina, PSA labeling was especially seen in the outer retinal layers. During retinal regeneration, similar widespread staining patterns were observed with the two antibodies, but labeling appeared higher with anti-total-N-CAM antibody than with anti-PSA-N-CAM antibody. On the other hand, tenascin (Tn) expression was analyzed for the first time during retinal regeneration. At the early stages, brightly stained matrix fibers of abundant Tn accumulating in the eye cavity were seen close to the retinal rudiment cells, which suggested that Tn was secreted from these cells. Tn expression was seen nearly throughout the retinal regenerate during neurite migration and then became restricted to the plexiform layers. In the light of the functions attributed to N-CAM and Tn in histogenetic events, the putative roles played by these morphoregulatory molecules in adult newt retinal regeneration were discussed.

KEY WORDS: *retina regeneration, N-CAM, tenascin, GFAP, urodele amphibians*

Introduction

The retina of a number of vertebrates including embryonic chick, frog tadpoles, but also adult teleost fishes and adult urodele amphibians is capable of neuronal regeneration (see e.g. review by Hitchcock and Raymond, 1992). Experimental morphological studies on different species of adult newts have revealed that retinal replacement originated from two cellular sources: (1) cells in the region of the *ora serrata* and *pars ciliaris retinae*, to which Keefe (1973a) referred to as "the anterior complex", and (2) the retinal pigmented epithelium (RPE) or *stratum pigmenti retinae* (see e.g. review by Stroeva and Mitashov, 1983). Cellular events of neural retina regeneration from the RPE through drastic phenotypic changes (a transdifferentiation process) including cell depigmentation, dedifferentiation and proliferation, have been examined in detail (Stone, 1950; Hasegawa, 1958; Mitashov, 1968; Reyer, 1971, 1977; Keefe, 1973a,b; Levine, 1975; Yamada, 1977). Autohistoradiographic studies using ^{3}H-thymidine deoxyribose (^{3}H-TdR) (Mitashov, 1968; Stroeva and Mitashov, 1970; Reyer, 1971; Keefe, 1973a) and ^{3}H-dihydroxyphenyl-alanine (^{3}H-DOPA) as a specific precursor of melanin synthesis (Mitashov, 1976, 1980; Grigoryan and Mitashov, 1979) enabled us to show that the RPE cells in the central area of the *fundus oculis* alone produce the retinal rudiment, and then, to determine the possible number of cells taking part in this process. The origin of neural retinal rudiment cells from the RPE cells was then specified by RPE-1, a monoclonal antibody of RPE cells in adult newts (Klein *et al.*, 1990). Using immunohistochemistry, it has also been shown (Ortiz *et al.*, 1992) that extracellular matrix (ECM) molecules, fibronectin, laminin, heparan sulfate proteoglycans and nidogen-entactin, did change during neural regeneration, suggesting that alterations in their composition might be important in the transdifferentiation of RPE cells into a new retina. Analysis of opsin mRNA

Abbreviations used in this paper: RPE, retinal pigmented epithelium; CNS, central nervous system; PNS, peripheral nervous system; ECM, extracellular matrix; Tn, tenascin; GFAP, glial fibrillary acidic protein; anti-total N-CAM PAb, polyclonal antibodies against all N-CAM isoforms; PSA, polysialic acid; PSA-N-CAM, polysialylated neural cell adhesion molecule; anti-PSA-N-CAM MAb, monoclonal antibody to capsule PSA units of Meningococcus B.

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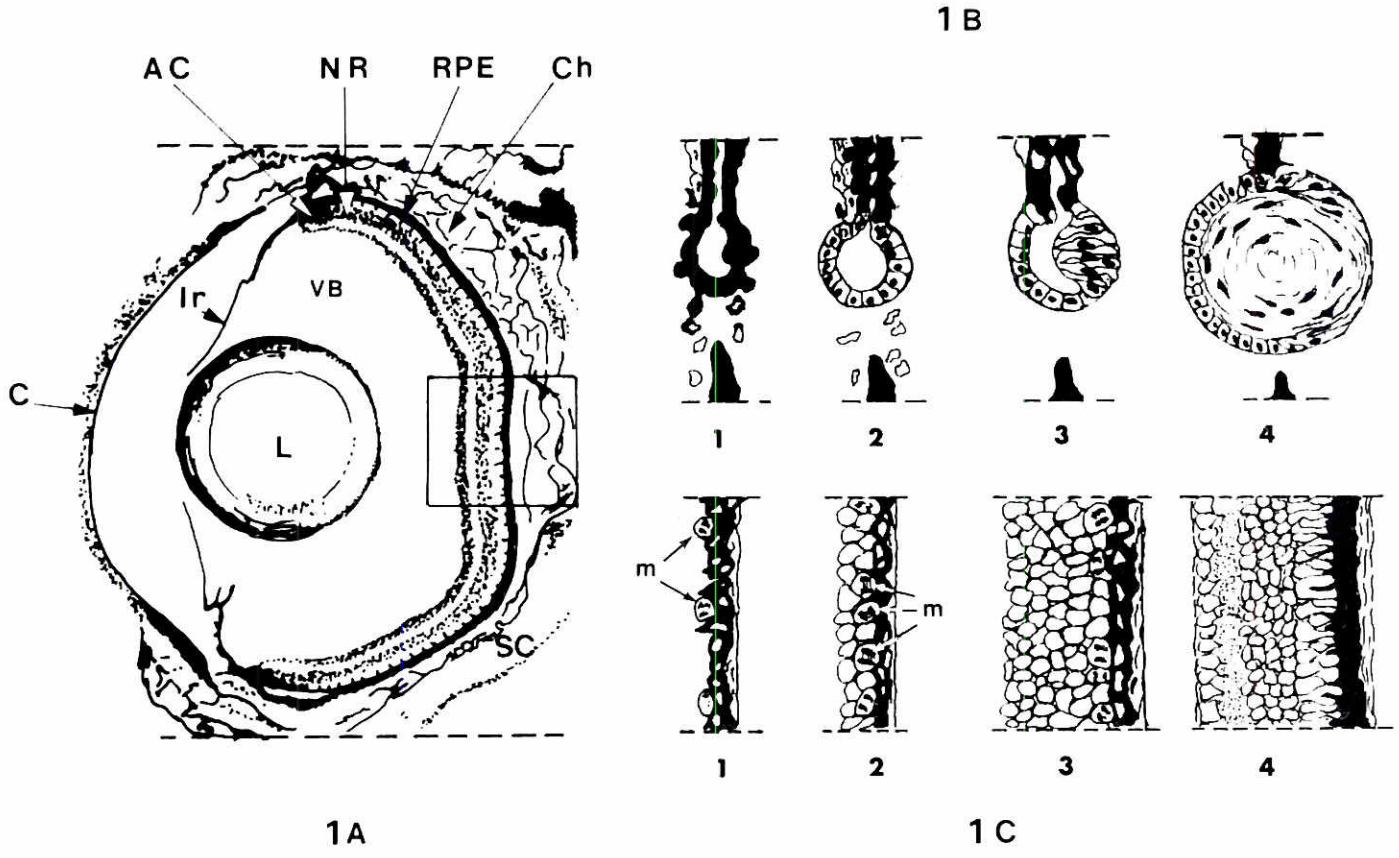


Fig. 1. Schematic representations of the normal eye and the regeneration stages of lens and retina in the adult newt. (A) *Vertical, meridional section through the adult normal eye. NR, neural retina; RPE, retinal pigmented epithelium; Ch, choroid; SC, sclera; VB, vitreous body; L, lens; Ir, iris; AC, anterior complex with ora serrata and ciliary epithelium; C, cornea.* **(B)** *Lens regeneration stages (1-4). Each step is represented in a section through the dorsoventral axis of the iris. (1-2) 10-15 days after lentectomy. Formation of a vesicle of depigmented epithelial cells from the inner and outer laminae of dorsal iris. (3) 20 days after lentectomy. (4) 30-35 days after lentectomy. Regenerating lens containing fibers.* **(C)** *Retinal regeneration stages (1-4) from the RPE. Only parts of retinal regenerates corresponding to the normal adult retinal central area enclosed in* **(A)** *are presented. (1) 5-10 days after retinectomy. Dedifferentiation and proliferation of the RPE cells begin. m, mitotic cells. (2) 14 days after retinectomy. Double-layered retinal rudiment lying next to a monolayer of repigmented epithelial cells. (3) 20 days after retinectomy. Retinal regenerate appears multilayered but no differentiated layers can be seen. (4) 30-35 days after retinectomy. Retinal regenerate displays differentiated cell and fiber layers. (Schemes drawn from Reyer, 1977; Eguchi, 1979 and our observations).*

and protein expression in adult and regenerating newt retinae using a combination of immunochemical and molecular biological approaches, enabled Bugra *et al.* (1992) to show that the redifferentiation of rod photoreceptors is a relatively late event.

On the other hand, it is now well known that, in vertebrates, during CNS or PNS development, but also in some regeneration processes, many histogenetic events involve cell-cell and cell-substrate interactions largely mediated by Neural Cell Adhesion Molecules (N-CAM) (for review see e.g. Edelman, 1985) and Tenascin/Cytotactin/J1 (for reviews, see Edelman, 1986; Chiquet, 1989; Erickson, 1993; Wehrle-Haller and Chiquet, 1993). The extent of glycosylation has been suggested to regulate functions of N-CAM (Edelman, 1986), the presence of Polysialic Acid (PSA) on the molecule providing broad steric effects (Yang *et al.*, 1992) which could modulate both cell-cell (Rutishauser, 1989) and cell-substrate interactions (Landmesser *et al.*, 1990). The conversion from a polysialylated (PSA-N-CAM) to a weakly sialylated (N-CAM) isoform has been shown to be generally correlated with a loss of tissue plasticity and a stabilization of adhesion. In relation with its properties of less adhesivity, PSA-N-CAM has been involved in dynamic morphogenetic events of development or regeneration. For example, PSA-N-CAM remains expressed in particular areas of the brain of adult mammals in which it may be implicated in neuroplasticity, continuous renewal of cells and cell reshaping (see e.g. Bonfanti *et al.*, 1992). On the other hand, it has been previously reported (Caubit *et al.*, 1993) that, during newt tail regeneration, remodeling processes of the ependymal tissue from which the spinal cord regenerates in adult urodeles, might be correlated with a transient reexpression of PSA-N-CAM. In the visual system, as pointed out by Bartsch *et al.* (1990), antibodies to N-CAM have been shown to disturb retinal histogenesis (Buskirk *et al.*, 1980) in regeneration as well as in development of the optic pathways. Bartsch *et al.* (1989, 1990) investigated the expression patterns of PSA during development of the retinotectal system in mice. In the developing embryonic and postnatal mouse retina, PSA was detectable in all cell types whereas it continued to be only expressed by astrocytes and Müller cells in the adult mouse

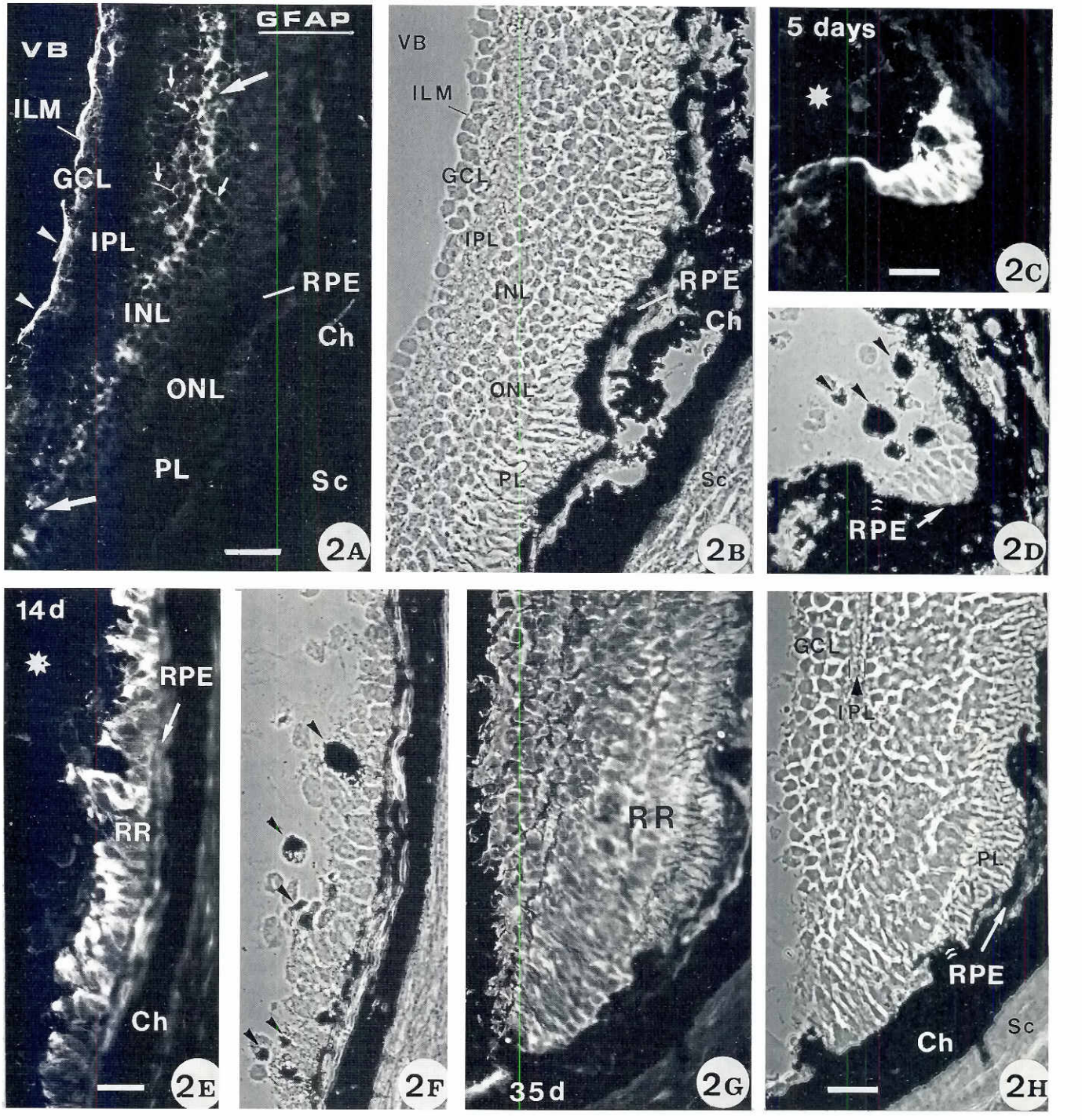


Fig. 2. GFAP labeling in normal and regenerating retinae of adult newt. **(A-B)** *Part of radial section through unoperated retina.* **(A)** *Müller cells (large arrows) and their fine processes (small arrows) are stained. High labeling seen in cell profiles (arrowheads) along the internal limiting membrane (ILM) or in the ganglion cell layer (GCL) may be Müller endfeet and/or astroglial cells supporting ganglional axons. VB, vitreous body; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; PL, photoreceptor layer; RPE, retinal pigmented epithelium; Ch; choroid; Sc, sclera.* **(B)** *Phase-contrast of* **(A)**. *Note that the outer plexiform layer is not visible here.* **(C-D)** *5 days after retinectomy.* **(C)** *Cluster of presumptive retinal progenitor cells displaying strong staining. Star, eye cavity.* **(D)** *Phase-contrast of* **(C)**. *Arrowheads, pigmented cells.* **(E-F)** *14 days after operation.* **(E)** *Retinal regenerate (RR) formed of 1 or 2 layers of cells showing reactive sites. Star, eye cavity.* **(F)** *Phase-contrast of* ***(E)***. *Arrowheads indicate pigmented cells/melanophages.* **(G-H)** *35 days post-surgery. Retina appears multilayered but here not yet fully differentiated.* **(G)** *A fine labeling seems to be present over all layers.* **(H)** *Phase-contrast of* **(G)**. *Bar, 50 μm.*

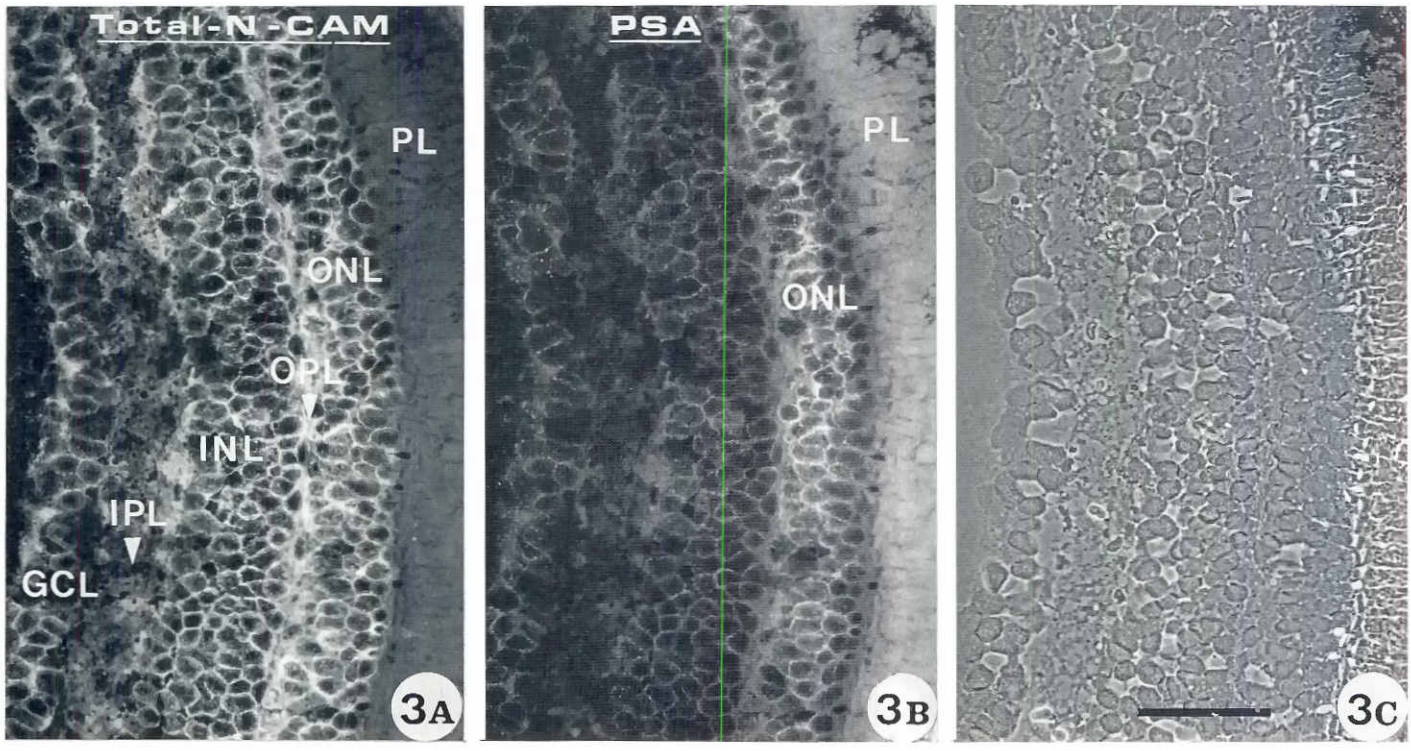


Fig. 3. Double staining with total-N-CAM and PSA-N-CAM Ab in normal retina of adult newt. (A) *Total-N-CAM localization. Intense labeling is consistently detected around the cells and over the fiber layers, except on the photoreceptor layer (PL). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.* **(B)** *PSA-N-CAM localization. High staining is observed around the cells of the ONL and over the photoreceptor layer (PL) whereas a weaker labeling is seen in the other retinal layers.* **(C)** *Phase-contrast of micrographs* **(A)** *and* **(B)**. *Bar, 50 μm.*

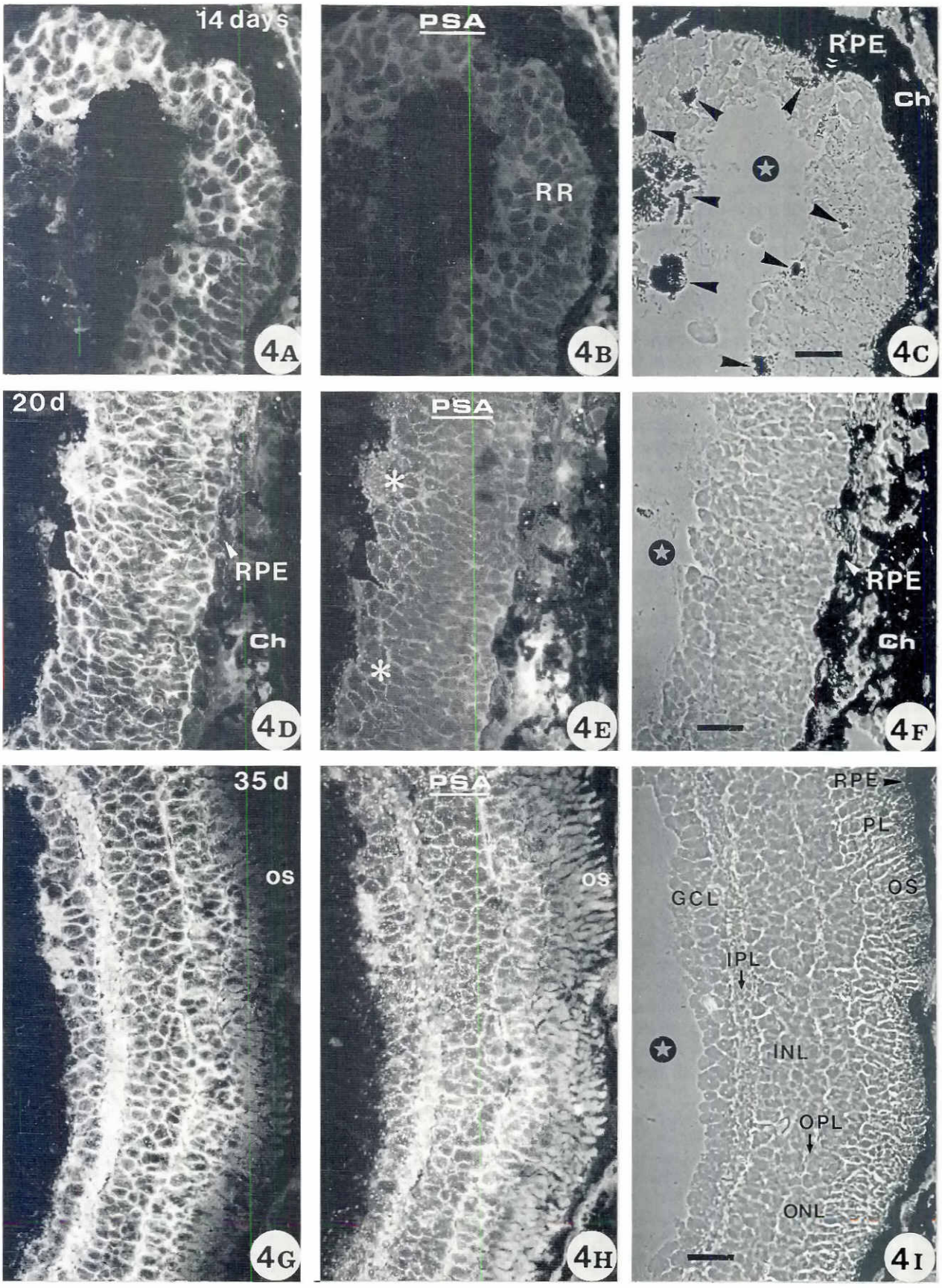
optic nerve and retina. Furthermore, more recently, Becker *et al.* (1993) claimed that the temporal and spatial distribution of PSA in retina of *Pleurodeles waltl* was *unusual* because it was low in the developing retina and high in the adult. These workers showed however that PSA was selectively downregulated in the adult optic nerve, and was *not* reexpressed during regeneration following crush-lesion. It is noteworthy that N-CAM isoform expression, during regeneration of the neural retina, was not examined in the paper by Becker *et al.* (1993); this was the reason why we considered this point in the present issue.

On the other hand, we postulated that tenascin (Tn) might be implicated in neural retinal regeneration of adult newts since this molecule plays an important role, not only in the development of the CNS (Grumet *et al.*, 1985; Kruse *et al.*, 1985; Crossin *et al.*, 1986; Chiquet, 1989; Chiquet *et al.*, 1991; Wehrle-Haller and Chiquet, 1993), especially in that of retina (Crossin *et al.*, 1986; Tucker, 1991; Perez and Halfter, 1993), but also in regeneration (Caubit *et al.*, 1994), including that of nerves (Daniloff *et al.*, 1989). The decrease of Tn expression during embryonic development (Crossin *et al.*, 1986), and its increase upon nerve injury (Daniloff *et al.*, 1989) could be correlated with cell migration events which are known to occur in these two kinds of processes. Although the role of Tn remains under debate (see e.g. Erickson, 1993), especially because of its complex multidomain structure allowing amphitropic properties (see e.g. Prieto *et al.*, 1992), it seemed to us interesting to analyze Tn expression patterns during the adult newt retinal regeneration.

Finally, since Müller glial cells are the main supporting cells for the retina (Keefe, 1971), we thought that their visualization by GFAP antibody (Bignami, 1984) might be useful in order to obtain an overview of its reorganization pattern during regeneration.

Therefore, in this paper we have examined dynamic features of the adult newt retina during regeneration using antibody to

Fig. 4. Double-labeling with total-N-CAM and PSA-N-CAM Ab in retinal regenerates. (A-C) *14 days after retinectomy. Staining is observed around all the cells of the retinal regenerate (RR), but it is high with total-N-CAM PAb* **(A)** *whereas it is weak with PSA-N-CAM MAb* **(B). (C)** *Phase-contrast image of* **(A)** *and* **(B)**. *Note that pigmented cells (arrowheads) are still present. RPE, retinal pigmented epithelium. Ch, choroid; star, eye cavity.* **(D-F)** *20 days after retinectomy. Retinal regenerate appears multilayered but not fully differentiated.* **(D)** *All cell surfaces are intensely stained with total-N-CAM PAb.* **(E)** *Only cell surfaces in the innermost layers (asterisks) of retina appear clearly labeled with PSA-N-CAM MAb.* **(F)** *Phase-contrast of* **(D)** *and* **(E)**. **(G-I)** *35 days after rectinectomy. The newly formed retina shows layering nearly comparable to that of the normal eye. Widespread labeling is observed with total-* **(G)** *and PSA-* **(H)** *N-CAM Ab. All cell and fiber layers are double-stained, except the photoreceptor outer segments (OS) which are PSA-positive.* **(I)** *Phase-contrast of* **(G)** *and* **(H)**. *GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PL, photoreceptor layer. Bar, 50 μm.*


14 days
4A
PSA
RR
4B
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Ch
4C
20d
RPE
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4D
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RPE
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35 d
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4G
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os
4H
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PL
OS
GCL
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INL
OPL
ONL
4I

GFAP as a marker of glial cells to follow the regeneration stages, and antibodies against total-N-CAM, PSA-N-CAM or Tn to analyze the modifications of cell-cell and/or cell-ECM interactions.

Results

Histological data

As reported above, after surgical removal from the adult newt eye (Fig. 1A), the neural retina and the lens are completely regenerated from the RPE and the pigmented epithelium of the dorsal pupillary margin of the iris, respectively.

Although lens regeneration has not been examined here, the main steps of this process are briefly represented in Figure 1B (scheme drawn from Reyer, 1977; Eguchi, 1979 and our observations).

To facilitate the interpretation of immunohistochemical data given in this paper, the schedule of events taking place during retinal regeneration of the adult newt eye has been represented in Figure 1C (scheme drawn from Reyer, 1977; Eguchi, 1979 and our observations) and can be summarized as follows:

- 5 to 10 days after retinectomy, in the central part of the fundus oculi, dedifferentiation and proliferation of the RPE cells begin (Fig. $1C_1$). The morphological features of this dedifferentiation process may be easily followed by the depigmentation of the RPE cells.

- 10 to 15 days after retinectomy, one- or two-layered retinal rudiment can be seen lying next to the RPE (Fig. $1C_2$, corresponding to Figs. 2F, 4C, 5F).

- About 20 days after retinectomy, retinal regenerate appears multilayered but not yet differentiated (Figs. $1C_3$ and 4F). The cells of the outer layer close to the Bruch's membrane redifferentiate to give repigmented epithelial cells. Differentiation of the new retina proceeds then from the inner to the outer layers.

- 30 to 35 days post-surgery, the newly regenerated retina (Figs. $1C_4$ and 2H, 4I, 5H) shows layering nearly comparable to that of the normal adult retina (Fig. 3C). This multilayered organization pattern of retina consists of the following layers, from the inner to the outer ones: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer (PL) and retinal pigmented epithelium (RPE).

GFAP expression

In the unoperated eye of the adult newt, the neural retina (Fig. 2B) displayed bright GFAP staining (Fig. 2A). This labeling seemed to concern especially Müller glial cells, whose nuclei were located in the inner nuclear layer and their fine cytoplasmic extensions (Fig. 2A). Immunoreactive profiles seen along the inner limiting membrane or just below, in the ganglion cell layer (Fig. 2A) could be Müller cell endfeet or more likely astrocytes supporting retinal ganglional axons. Intense GFAP labeling of the optic nerve (result not shown) was also probably due to this peripheral astroglial system.

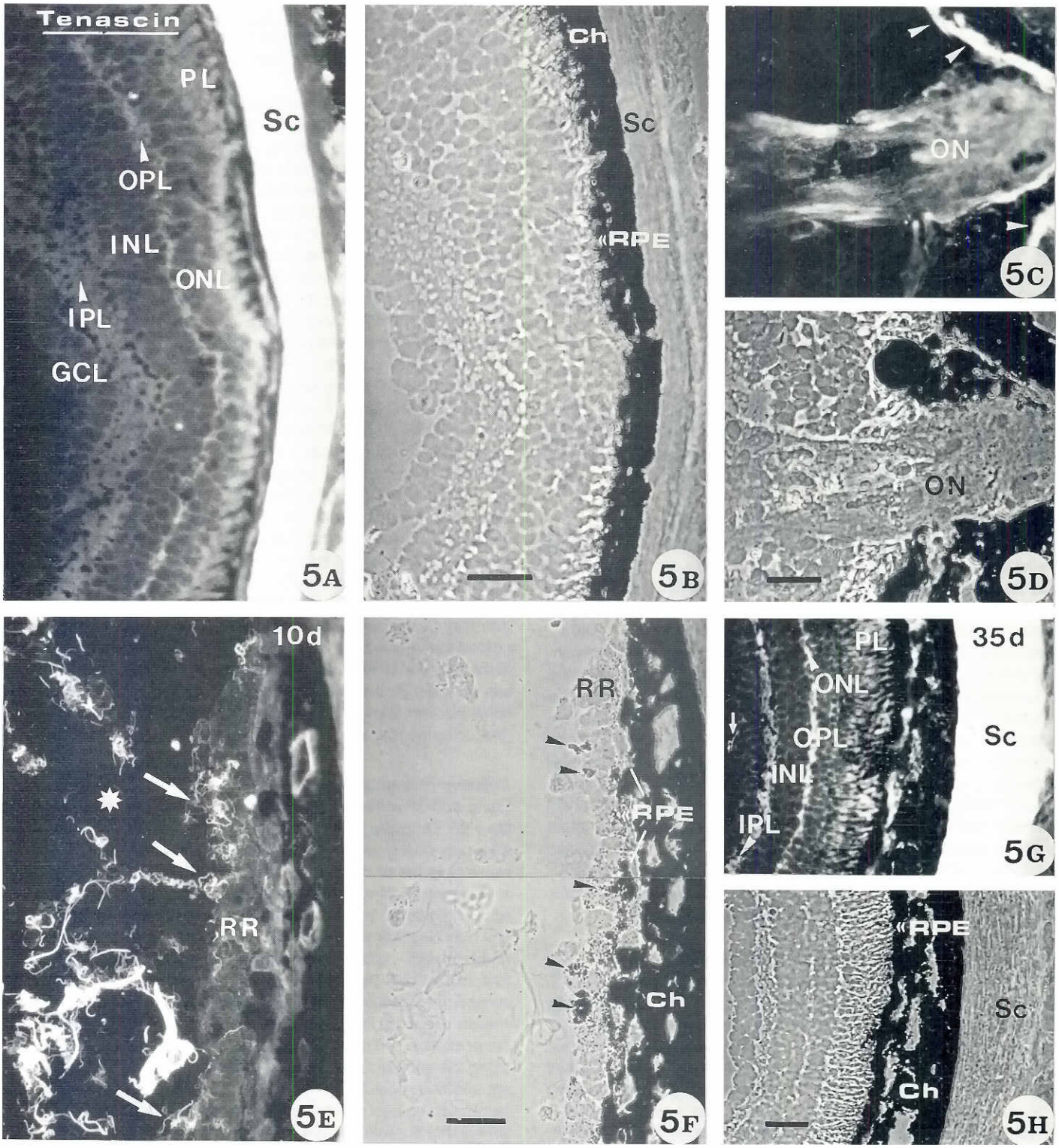
In the early stages of retinal regeneration, i.e. as soon as 5 days following retinectomy (Fig. 2 C/D), clusters of presumptive retinal progenitor cells, which were neuroepithelial cells originating from pars ciliary/ora serrata complex, displayed high GFAP staining. By 10-14 days post-retinectomy, a single, double or triple-layered retinal regenerate originating from the retinal pigmented epithelium was generally established upon Bruch's membrane (Fig. 2F). Although most of new retinal cells were depigmented, scattered pigmented cells were still present in the retinal rudiment and in the eye cavity (Fig. 2F). Most cells forming this retinal anlage were oriented perpendicular to the pigmented layers (Fig. 2F) and exhibited intensely reactive sites (Fig. 2E). About 35 days after retinal removal, the newly reconstituted retina showed a multilayered organization pattern (Fig. 2H). It was however not yet fully differentiated, the central region being better defined than flanking zones extending to the ciliary margins (Fig. 2H). At this retinal regeneration stage, a fine GFAP labeling could be seen over all the differentiating cell and fiber retinal layers (Fig. 2G). GFAP immunoreactivity became later (results not shown) restricted to Müller glial cell and radial processes and astroglial cells associated with retinal ganglion cell axons, like it was observed in the normal adult retina (Fig. 2A).

Expression of total-N-CAM and PSA-N-CAM

Double immunolabeling experiments with anti-total-N-CAM and anti-PSA-N-CAM antibodies were performed on cryosections through normal and regenerating retinae. In normal retina of post-metamorphic newts (Fig. 3C), strong immunoreactivity with anti-total N-CAM PAb was observed widespread throughout the cell and fiber layers, except the photoreceptor layer (Fig. 3A). Although a lower staining with anti-PSA-N-CAM MAb was seen in most of the normal retinal layers, high PSA labeling was observed around the cells of the outer nuclear layer and over the photoreceptors (Fig. 3B).

In 1 to 2-week-old retinal rudiments which still contained scattered pigmented cells (Fig. 4C), total-N-CAM appeared intensely expressed (Fig. 4A) but PSA-N-CAM faintly expressed (Fig. 4B) around the new retinal cells. A similar difference in staining patterns with anti-total-N-CAM PAb (Fig. 4D) and anti-PSA-N-CAM MAb (Fig. 4E) was observed in 3-week-old regenerates (Fig. 4F). A bright total-N-CAM reactivity was seen at the retinal cell surfaces (Fig. 4D). Only a weak PSA labeling was detected (Fig. 4E). This PSA staining seemed to be slightly higher around the cells located in the inner part of the retinal rudiment (Fig. 4E). In 35-day-old regenerates, all the cell and fiber retinal layers, which were now well identifiable (Fig. 4I),

Fig. 5. Tenascin localization in adult unoperated and regenerating retinae. (A-B) *Part of radial section through normal retina.* **(A)** *Staining is observed on the inner (IPL) and outer (OPL) plexiform layers. Note the bright staining of the sclera (Sc). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PL, photoreceptor layer.* **(B)** *Phase-contrast of* **(A)**. *RPE, retinal pigmented epithelium; Ch, choroid.* **(C-D)** *Section through the adult optic nerve (ON) head showing that it is intensely stained. Arrowheads indicate fluorescent Bruch' s membrane and/or choroid.* **(E-F)** *10 days after removal. One- or two-layered retinal regenerate (RR) lying next to the RPE.* **(E)** *Rudiment retinal cells are unlabeled or weakly labeled, but some of the stained fibers in the eye cavity (star) appear locally (arrows) close to them.* **(F)** *Phase-contrast of* **(E)**. *Note that pigmented cells (arrowheads) are still present.* **(G-H)** *35 days after retinectomy. The newly formed retina appears multilayered and well differentiated.* **(G)** *High labeling is seen on the plexiform layers and over the sclera. A presumptive developing ganglional axon (arrow) also displays staining.* **(H)** *Phase-contrast of* **(G)**. *Bar, 50 µm.*



displayed nearly similar expression patterns to the two anti-N-CAM antibodies, i.e., with anti-total-N-CAM PAb (Fig. 4G) and with anti-PSA-N-CAM MAb (Fig. 4H). All cell and fiber retinal layers were intensely double-stained (Fig. 4 G and 4 H), except the layer of the photoreceptor outer segments which was only PSA-reactive (Fig. 4H).

Tenascin (Tn) expression

In the unoperated eye of the adult newt, the optic nerve (Fig. 5C/D) and many other components, such as corneal stroma and Descemet's membrane, lens capsule and iris (results not shown), choroid and sclera (Fig. 5A/B) showed Tn immunoreactivity. In the neural retina, Tn expression seemed

to be nearly restricted to the inner and outer plexiform layers (Fig. 5A/B).

About 10 to 15 days after retinectomy, the simple or double-layered retinal rudiments lying close to the pigmented epithelium (Fig. 5F) displayed no conspicuous Tn labeling (Fig. 5E). However, interestingly, some intensely reactive twisting fibers present within the vitreous body occurred in topographic association with cells of these retinal regenerates (Fig. 5E/F). Later, e.g. in 35-day-old retinal regenerates, which showed a complex laminar but not yet fully differentiated pattern (Fig. 5H), Tn appeared nearly expressed throughout all the cell and fiber layers (Fig. 5 G). It is noteworthy that high Tn expression was especially found at this stage in the inner and outer plexiform layers, and in the ganglional axons migrating along the optic fiber layer (Fig. 5G).

Discussion

In this paper, using immunohistochemistry, we have been able to specify some spatiotemporal dynamic features of neural retinal regeneration in the adult newt *Pleurodeles waltl*. Our main findings may be summarized and discussed as follows.

GFAP: a marker of retinal glial and progenitor cells

To have an overview of the reorganization pattern of the newt retina during regeneration, we used antibodies against GFAP as cell markers of glial cells to examine the positioning and reestablishment of the Müller supportive system whose extensive ramification was first described in newts by Keefe (1971). Unexpectedly, during this study, we observed that retinal progenitor (neuroepithelial) cells, thought to originate first from the pars ciliary/ora serrata complex, and then from the RPE cells, also displayed GFAP staining. Although GFAP has been considered as a specific marker for astrocytes (Bignami, 1984), there are reports demonstrating that GFAP is also widely expressed in other cell types, such as ependymal cells of the newt spinal cord (Arsanto *et al.*, 1992), immature and non-myelin-forming Schwann cells (Jessen and Mirsky, 1984) or cells of non-neuroectoderm origin such as developing lens epithelial cells of few animal species (Hatfield *et al.*, 1984). Therefore, our study suggested that GFAP antibodies, which stained retinal glial cells, especially Müller cells, might also be used to identify retinal progenitor cells.

Implication of total-N-CAM and PSA-N-CAM

We reported here the spatiotemporal distribution of total-N-CAM and PSA-N-CAM during regeneration of the neural retina in *Pleurodeles waltl*. In agreement with Becker *et al.* (1993), we found a sustained high expression of total-N-CAM in the adult newt retina. On the other hand, we observed a widespread expression of total-N-CAM in the regenerating retina of adult *Pleurodeles* at all developmental stages, as it was previously seen in the developing retina of larva (Becker *et al.*, 1993). Moreover, our study gave evidence that, in the adult newt eye, retinal regeneration involved PSA reexpression whereas optic nerve regeneration did not (Becker *et al.*, 1993). During adult newt retinal regeneration, although PSA clearly appeared lower than total-N-CAM staining, the two molecules roughly displayed similar expression patterns, which suggested that N-CAM was a carrier of PSA all along retinal restoration. However, we do not know the significance of PSA labeling seen on photoreceptor outer segments. It may be artefactual since proteins other than N-CAM were found to carry PSA (Zuber *et al.*, 1992).

On the other hand, it has been reported that PSA regulation in newts is directly correlated with metamorphosis (Becker *et al.*, 1993), which is, in amphibians, under control of thyroxine (Etkin, 1964). Furthermore, thyroxine is one of the hormones controlling newt limb and tail regeneration (Schmidt, 1968; Vethamany-Globus and Liversage, 1973). These data raise the question whether this hormone is also implicated in adult newt retinal regeneration. On the other hand, PSA-N-CAM was persistently expressed in the normal retina of adult newt, which was *unusual*, as remarked Becker *et al.* (1993). Indeed, PSA is generally downregulated in the adult CNS structures. Becker *et al.* (1993) suggested that the sustained expression of adhesion molecules such as N-CAM isoforms in the amphibian CNS might be due to paedomorphosis, an evolution and secondary simplification phenomenon which leads to a reduction or loss of terminal differentiation (Gould, 1977). According to Becker *et al.* (1993), paedomorphosis might explain the relatively low degree of differentiation shown by several amphibian nervous structures, including the retinotectal system of urodeles in which it is especially pronounced (Roth *et al.*, 1993). On the other hand, although PSA was downregulated in the adult mouse visual system, Bartsch *et al.* (1990) drove attention to the persistence of PSA-N-CAM expressed by Müller cell processes in the retina. On the basis that PSA was thought to be involved in synaptic plasticity (Aaron and Chesselet, 1989), Bartsch *et al.* (1990) suggested that these PSA-reactive glial cells might play a major role in mouse retinal synaptic rearrangement. It is tempting to postulate a similar role in plasticity for PSA-labeled cells which, in the outer nuclear layer of the adult newt retina, may also be Müller glial cell processes.

Putative role of tenascin

Using anti-Tn antibody, we analyzed, for the first time, the variations of Tn expression during adult newt retinal regeneration. Although young retinal regenerate exhibited no or only a faint Tn labeling, some of brightly Tn-stained matrix fibers in the eye cavity were seen close to the retinal rudiment cells, which suggested that Tn could be secreted from them. It is noteworthy that *in situ* hybridization studies led Tucker (1991) to the idea that the ciliary margin – which also contains multipotent precursor cells – was most likely the source of Tn accumulating in the vitreous body during development of the avian eye. Later, at the regeneration stage corresponding with time of retinal axon migration, the presence of Tn was detected on the developing optic axons along the ganglion cell layer, in the inner and outer plexiform layers and in a radial labeling through the retinal regenerate. A similar Tn expression pattern was observed by Crossin *et al.* (1986) during development of the retina in the chicken. *In vitro* studies on primary glial cells (Grumet *et al.*, 1985; Kruse *et al.*, 1985) and immunoelectron localization of Tn (Steindler *et al.*, 1989; Caubit *et al.*, 1994) implicated glial precursor cells and maturing astrocytes as possible major sources of Tn. Therefore, Tn present in the regenerating or developing retina, as neurite migration occurred, was most likely produced by Müller glial cells and astrocytes associated with ganglional axons.

Because antibodies against Tn were shown to inhibit neuron-glia adhesion *in vitro* (Grumet *et al.*, 1985; Kruse *et al.*, 1985), Tn was involved in neuronal-glial interactions during development of the CNS structures including the retina (Crossin *et al.*, 1986). However, more recent studies showed that Tn did not promote neurite outgrowth in several *in vitro* systems but, on the contrary, acted as a repulsive substrate for CNS neurons (Steindler *et al.*, 1989), including retinal axons (Perez and Hafter, 1993). The exact involvement of Tn in adhesion mechanism is far to be understood (see e.g. Chiquet *et al.*, 1991; Erickson, 1993; Wehrle-Haller and Chiquet, 1993). However, it is known that Tn is a multidomain protein with dual properties mediated by adhesive and anti-adhesive sites (Spring *et al.*, 1989; Lochter *et al.*, 1991; Prieto *et al.*, 1992). Based on the distribution of Tn and its neurite outgrowth inhibitory activity for retinal axons *in vitro*, Perez and Halfter (1993) proposed that Tn might play multiple roles in establishing the visual pathway. Tn could act in particular as a barrier for growing axons at the outer border of this pathway, i.e., specifically at the optic fissure, the optic nerve head and the optic chiasm. According many workers (for review, see e.g. Chiquet *et al.*, 1991), during regeneration as well as development, Tn might allow plasticity, facilitate axon fasciculation and help to confine neuronal pathway. On the other hand, the high density of Tn in the inner and outer plexiform layers at the late regeneration stages was consistent with the idea that Tn might be involved in the stabilization of synapses (Perez and Halfter, 1993). Furthermore, the role of Tn in wound healing (Mackie *et al.*, 1988), nerve regeneration (Daniloff *et al.*, 1989) and shaping of cytoarchitecture in the CNS is now well established. Taken together, all these data suggest that Tn may play a determinant part in retinal regeneration.

In conclusion, immunolabeling data presented in this paper provided new information about the reorganization pattern of the adult newt retina during regeneration:

1) Restoration of the retina could be roughly followed by GFAP staining of the Müller glial system. Furthermore, our study showed that the progenitor retinal cells expressed GFAP, which might be used as a marker of these cells.

2) Total-N-CAM and PSA-N-CAM were persistently expressed in the adult retina and displayed parallel developmental expression patterns during retinal regeneration.

3) Tn expression was seen nearly throughout retinal regenerates at the time of neurite migration. Then, it decreased and became restricted to the plexiform (synaptic) layers.

Therefore, given the key role attributed to N-CAM isoforms and Tn in histogenesis, our findings supported the view that these molecules might be involved in morphogenetic events of the adult newt retinal regeneration, e.g., in controlling axonal growth and synapse stabilization.

Materials and Methods

Animal surgery procedure

The Urodelan amphibia used in this study were adult newts of the species *Pleurodeles waltl*, obtained from the CNRS's Amphibian Farm, Centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France. Animals were reared in groups of 10-12 and maintained in circulating tap water at 18-20°C; the water was completely renewed twice a week. *Pleurodeles* were fed twice a week with beef heart or liver. Before lens and retina removal, animals were anesthetized by placing them in a 1% aqueous solution of MS 222 (tricaine methane sulfonate, Sigma).

Lens and retina were removed by cutting through the dorsal part of the eye. The cut was made in the contact area between the pigmented epithelium and the root of the dorsal part of iris. A physiological solution was injected between the pigmented epithelium layer and retina to obtain the detachment of one tissue from the other. The whole retina was removed by forceps after cutting the optic nerve. Six eyes were dissected for each stage of lens and retina regeneration.

After appropriate periods of regeneration (i.e., 5, 10, 14, 20, 25, 35 or 40 days following operation), the regenerating eyes were removed by microsurgery.

Antibodies

In this study we used polyclonal (PAb) and monoclonal (MAb) antibodies directed to cell adhesion molecules, extracellular matrix (ECM) and cytoskeletal components:

Anti-PSA-N-CAM (Polysialylated Neural Cell Adhesion Molecule) MAb

It is a mouse immunoglobulin M (IgM), raised against the capsular polysaccharides of *Meningococcus* group B (so-called anti-Men-B: Rougon *et al.*, 1986; Bartsch *et al.*, 1990; Bonfanti *et al.*, 1992), that shares α-2,8-linked N-acetylneuraminic (polysialic) acid residues. This antibody, which was a gift of Dr. G. Rougon, specifically recognizes the highly sialylated form of N-CAM (Rougon *et al.*, 1986).

Anti-total N-CAM PAb

A site-directed rabbit serum that recognizes the NH2-terminal residues of N-CAM sequence, which is shared by all isoforms (Rougon and Marshak, 1986), and an anti-chicken total N-CAM PAb (a gift of Dr. J-P. Thiéry). Cross-reactivity of anti-PSA-N-CAM and anti-total N-CAM antibodies with urodele amphibian antigens was recently demonstrated by some of us (Caubit *et al.*, 1993) both by Western blotting and indirect immunofluorescence.

Anti-tenascin PAb

It was prepared using *Xenopus laevis* tenascin purified from XTC cell line as immunogen (Riou *et al.*, 1991). Its cross-reactivity with urodele amphibian antigens was previously controlled by immunoblotting and immunohistochemistry (Caubit *et al.*, 1994; Arsanto *et al.*, 1995).

Anti-GFAP (Glial Fibrillary Acidic Protein) PAb

In the central nervous system, this polyclonal antiserum (Dakopatts Corp., CA, USA), directed against GFAP, specifically labels astrocytes (Bignami, 1984), including Müller cells, which are the main supporting cells for the neural retina (Keefe, 1971). The cross-reactivity of this antibody with urodele antigens was previously controlled by Western blots and immunohistochemistry (Arsanto *et al.*, 1992).

Immunohistochemistry

Samples were directly embedded unfixed in OCT Compound (Tissue Tek, Miles Laboratories Inc., Naperville, IL, USA). They were rapidly frozen in liquid nitrogen and stored at -20°C. Serial sections of 15 µm were obtained in a cryostat at -22°C, collected on gelatined slides and stored at -20°C until immunofluorescent staining was performed. They were washed 1 h in PBS+1% bovine serum albumin (BSA), then incubated for 1 h in a humid chamber in the dark, with primary antibodies used at a 1:50 or 1:100 dilution. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or anti-mouse IgG or IgM were used at a 1:100 or 1:200 dilution as secondary antibodies. Washed slides were mounted in moviol and observed with an epifluorescence Zeiss microscope and photographed on Tri-X pan (Kodak). Controls were made by omitting the first antibody or by replacing it with preimmune serum.

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