Accumulation of Gliol Fibrillary Acidic Protein in Müller Radial Glia During Retinal Degeneration

P. Ekström,* S. Sanyal,† K. Narfsström,‡ G. J. Chader,§ and T. von Veen*

Müller radial glia accumulate glial fibrillary acidic protein (GFAP) in response to retinal injuries. We have studied the changes in cellular localization of GFAP in genetically caused retinal dystrophy in strains of cat and mouse: Abyssinian cats with progressive retinal dystrophy, and mice homo- and heterozygous for the retinal degeneration (rd) and retinal degeneration slow (rds) genes. The following observations were made: (1) Gliol fibrillary acid protein-immunoreactive (GFAP-IR) radial Müller glia are present in normal cat and mouse retinae. (2) There is a general increase with age in numbers of GFAP-IR radial Müller glia, and other GFAP-IR elements in the retina of cat and mice with hereditary retinal dystrophy. (3) The increase in GFAP-accumulating Müller cells seems to proceed from peripheral to central retina in both cats and mice. This might reflect the direction of photoreceptor degeneration, which proceeds in the same direction in the retinal dystrophic cat and in mice bearing the rds gene. However, in the rd mouse photoreceptor degeneration proceeds from central to peripheral retina, indicating that GFAP accumulation is not a local direct effect of photoreceptor damage. (4) Comparing the different mutant mouse strains, there seem to be qualitative differences in the distribution of GFAP-IR elements. The numbers of tangential elements and fibrillary tangles in the inner plexiform and nuclear layers are highest in the +/+ rds/rds retina, followed by the +/+ rds/+, whereas such elements appear to be more scarce in rd/rd rds/rds, followed by the rd/rd +/+ and +/+ +/+ retinal retinae. (5) Accumulation of GFAP-IR elements occurs at the earliest stages in the rd/rd +/+ mouse, followed by the rd/rd rds/rds, the +/+ rds/rds, +/+ rds/+, and the normal mouse, in agreement with the onset and time course of the degenerative changes. Invest Ophthalmol Vis Sci 29:1363–1371, 1988

The intermediate filament protein, glial fibrillary acidic protein (GFAP, MW 49 kD) forms the major cytoskeletal component of mature astrocytes in the central and peripheral nervous systems of mammals. During development, expression of GFAP in retinal astrocytes is preceded by expression of vimentin, an intermediate filament protein specific for immature glia and cells of mesenchymal origin. In the radial glia of the retina, the Müller cells, the early expression of vimentin is retained throughout life. In the adult normal retina, Müller cells do not contain GFAP however, as a result of injury to the retina Müller cells may accumulate GFAP. Elucidation of the patterns of GFAP accumulation may have implications for the understanding of: (1) (epi)genetic control of intermediate filament differentiation; (2) regulation of cell differentiation during developmental retinal disease; and (3) neuron-glia interaction in the retina. In this study we have followed the changes in the cellular localization of GFAP during the course of different hereditary retinal degenerations in cat and mouse strains. In a strain of Abyssinian cats with progressive retinal atrophy (PRA), an autosomal recessive mutation has been shown to result in the progressive loss of photoreceptor cells following their apparently normal development. In mice, two different mutations are known to cause degeneration and...
Results.

Animals were killed under ether anaesthesia. Gray lesions on either side of the optic disc), and the only in a few cases. Animals of different ages, be-

The anterior segments removed and the eyecups fixed 4, with generalized hyperreflectivity of the tapetal paraformaldehyde. Sections from selected stages of the various mu-

tants and comparable control eyes, have been pro-
cessed for immunocytochemistry using GFAP anti-
sersum. The time course for the topographical changes in the immunoreactions has been compared with the progress of the retinal lesions.

Materials and Methods

Experimental Animals

Abyssinian cats homozygous for progressive retinal atrophy (PRA) were studied at 12 weeks, 10 months and 7 years of age. The degree of retinal degeneration was assessed by ophthalmoscopy and/or ERG measure-

ment and staged according to Narfström. Based on these evaluations, the 12-week-old animal was considered normal (stage 0), the 10 months animal had early photoreceptor disease (stage 1, with slight gray lesions on either side of the optic disc), and the 7-year-old animal had advanced degeneration (stage 4, with generalized hyperreflectivity of the tapetal fundus and vascular attenuation).

As controls, normal Abyssinian cats of 12 weeks and 1.5 years age (one animal of each age) obtained from the same laboratory animal facility were used. From each of the cats, one eye was surgically re-
moved under anesthesia at ages indicated above, and the anterior segments removed and the eyecups fixed overnight by immersion in 4% phosphate-buffered paraformaldehyde.

Two strains of mice, pigmented C3H and Albino Balb/c bearing congenic lines with the following ge-

notypic combinations had been produced and pro-
vided the materials in this study: +/+, +/+ (control), rd/rd +/+, rd/rd +/+, rd/rd +/+, rd/rd +/+, rd/rd +/+, rd/rd +/+, and rd/rd +/+. Most of the investigations were performed on C3H strains of mice, whereas Balb/c mice were used only in a few cases. Animals of different ages, be-
 tween newborn and 18 months were used, as listed in Results. Animals were killed under ether anaesthesia. Whole eyes were immersed in the fixation fluid (as above), the cornea and the lens were removed within 5–10 min and fixation was continued over night. The tissues were rinsed in several changes of Tyrode buffer, infiltrated with Tyrode buffer containing 25% sucrose, embedded in Tissue-Tek cryoprotection me-
dium (Miles Scientific), and frozen on a metal plate cooled with liquid nitrogen. All specimens were seri-

ally sectioned on a Reichert-Jung cryostat (mouse retinae: 6 μm thick sections; cat retinae: 10 μm thick sections) and mounted on chrome alum gelatinized slides. After air-drying, slides were stored at −70°C until immunocytochemical processing.

All animals were treated according to the ARVO Resolution on the Use of Animals in Research.

Immunocytochemistry

After thorough rinses in wash buffer (Phosphate Buffered Saline, PBS, containing 0.25% Triton X-100) sections from each eye were incubated over night with rabbit anti-GFAP diluted 1:200. After 2 

× 10 min rinses in buffer, sections were incubated 30 min in swine anti-rabbit IgG diluted 1:50. Following 2 × 10 minute rinses in buffer, the sections were incubated 30 minutes in rabbit PAP complex diluted 1:50. All incubations, including the one with anti-
GFAP, were performed at room temperature (ca. 20°C). After buffer rinses, peroxidase activity was demonstrated with 3,3’-diaminobenzidine tetrahydro-
drochloride (0.05%) and hydrogen peroxide (0.015%) in TRIS-HCl buffer (pH 7.2). Sections were rinsed in buffer, dehydrated in an alcohol series, cleared in xy-
 lene and coverslipped with Permount mounting me-

dium. All antibodies were purchased from DAKO-
PATTS (Copenhagen, Denmark). All antibodies were diluted in wash buffer containing 1% bovine serum albumin. Since quenching of endogenous per-

oxidase activity (with hydrogen peroxyde in metha-

nol) did not influence the staining pattern otherwise than reducing erythrocyte labeling, this step was rou-

tinely omitted.

Results

Normal Cat Retina

In the 12-week-old normal kitten, radial Müller glia expressed glial fibrillary acidic protein-immuno-

reactivity (GFAP-IR). The endfeet of the Müller cells were strongly labeled, but GFAP-IR of the radial processes often could not be observed distal to the inner nuclear layer (INL) (Figs. 1, 2). A small number of immunoreactive Müller cell somata were found in the INL (Fig. 1). Similar findings were present in older (1.5 year) normal cat (Fig. 3), with somewhat larger numbers of radial fibers in the peripheral ret-

ina. GFAP-IR radial fibers were only rarely seen out-

side the optic axon layer (OAL). GFAP-IR was also detected in astrocytes in the OAL.
Affected Cat Retina

The retina of the 12-week-old (stage 0) affected kitten showed somewhat larger numbers of GFAP-IR radial elements, that sometimes extended into the outer nuclear layer (ONL) (Fig. 4). In addition, horizontal GFAP-IR elements occurred in the outer plexiform layer (OPL) and in the inner plexiform layer (IPL). Since it was not possible to discern whether the GFAP-IR elements in the OPL represent horizontal cells, or radial Müller cell processes, we prefer to use the term horizontal elements. GFAP-IR cell somata were observed in the INL, both in the middle and outermost cell rows.

In the 10-month-old retinal dystrophic (stage 1) cats (Fig. 5), degenerative changes were apparent as a reduction in the number of photoreceptor nuclei in the ONL. The GFAP-IR Müller cells were not evenly distributed, but tended to form groups, or clusters. In the vicinity of these groups, horizontal GFAP-IR ele-
ments were abundant in the IPL (Fig. 5). Highly disorganized tangles of GFAP-IR fibers were also observed in these areas (Fig. 5). In the central retina, close to the optic disc, a particularly strong proliferation of GFAP-IR fibers occurred. However, a characteristic of the disease at this stage is the presence of GFAP-IR radial fibers throughout the retina, and in the outer plexiform layer, horizontal GFAP-IR fibers were distinct.

The retina of the 7-year-old (stage 4) affected cat showed advanced degenerative changes. The overall thickness of the retina was greatly reduced, and the outer nuclear and plexiform layers had almost completely disappeared. GFAP-IR Müller cells dominated the retina (Fig. 6). Their radial processes were very coarse, and showed a repetitive branching pattern. Numerous immunoreactive cell bodies were observed in the inner nuclear layer, and GFAP-IR was also present in horizontal astrocytes of the OAL.

**Normal Mouse Retina**

GFAP-immunoreactive astrocytes were observed in the optic axon layer at all developmental stages studied, i.e., at P4, P14, P28, P4m, P8m and P18m. GFAP-IR Müller radial glial profiles were detected in very small numbers at P14 (Fig. 7) and P28. They occurred primarily in the peripheral retina, and their distal processes could sometimes be traced into the ONL. At P4m, an increase in number and immunoreactivity of GFAP-positive Müller cells was noted. GFAP-IR horizontal elements were observed in the outer plexiform layer at P14 (Fig. 7), P28, P4m, P8m (Fig. 8) and P18m (Fig. 9). As was the case for the cat retina, the identity of these horizontal elements could not be verified. They might represent horizontal cells but for want of conclusive evidence we describe them simply as horizontal elements. The retina of the 8-month-old mouse contained less GFAP-IR Müller cells in the central portion of the retina (Fig. 8) than at 4 months. In the very old (18 month) mouse, immunoreactive GFAP Müller cells were relatively evenly distributed throughout the retina (Fig. 9).

**rd/rd,+/+ Mutant Mouse Retina**

At P7 GFAP-IR elements were limited to astrocytes in the optic axon layer and a small number of GFAP-IR horizontal fibers in the outer plexiform layer. At P11, the number and distribution of the GFAP-IR elements had increased with some radial fibers (Müller cells) becoming apparent and a diffuse labelling in the inner plexiform layer. In contrast to the normal, the P14 retina showed a dramatic increase in the number of strongly GFAP-IR Müller cells whose radial fibers were distributed throughout the entire retina including ONL (Figs. 10, 11). Strongly labelled GFAP-IR horizontal elements were present in the ONL.

At P21, the overall thickness of the retina was greatly reduced, mainly because the ONL consisted of only one or two rows of nuclei. Large numbers of strongly GFAP-IR Müller cells occurred throughout the retina, but the radial fibers of individual cells did not appear to give rise to branches in the inner plexiform and nuclear layers. However, strongly GFAP-IR horizontal elements were observed throughout the outer plexiform layer.

Strongly GFAP-IR Müller cells that penetrated the entire retina and gave rise to processes covering the external surface were observed both at P2m (Fig. 12) and P6m. GFAP-IR astrocytes were observed in the optic axon layer of the central retina at all developmental stages.

**+/+, rds/rds Mutant Mouse Retina**

At P7 the only GFAP-IR elements were astrocytes in the optic axon layer. These elements could be identified at all developmental stages. At P11, small numbers of GFAP-IR radial Müller glia occurred along with horizontal fibers in the outer plexiform layer. Here, the strongest labeling was observed in the peripheral retina. This pattern was unchanged at P14 (Fig. 13). At P28, the number of GFAP-IR Müller cells had increased. There was a tendency towards an uneven distribution of these cells, as if they formed groups. Occasional fibrillar tangles were observed in the inner plexiform layer. The outer nuclear layer consisted of approximately eight rows of cell somata at this stage.

At P2m, the outer nuclear layer still retained approximately eight rows of cell somata. Inner segments appeared shorter than at P28. The density of GFAP-IR radial Müller glia had increased dramatically (Fig. 14). In the peripheral retina they gave rise to processes that penetrated the outer nuclear layer and covered it externally. The number of fibrillar tangles in the inner plexiform layer had increased somewhat, as had the density of GFAP-immunoreactive elements in the outer plexiform layer.

At P9m, the outer nuclear layer had decreased in thickness to 2 to 3 rows of cell somata. The density of GFAP-IR radial glia, as well as horizontal fibers in the plexiform layers and fibrillar tangles had increased further. Almost the entire external surface of the outer nuclear layer was covered by GFAP-IR Müller cell processes.

At the last stage investigated of this mutant strain, P18m (Fig. 15), it was not possible to discern a demarcated outer nuclear layer. At most, a single layer
Figs. 7-9. Normal (+/+ +/+ ) mouse retina at different postnatal (P) ages. Scale bar 50 μm. Fig. 7. P14d. Müller cell endfeet (arrowheads) and horizontal elements in the OPL (arrow) are GFAP-IR. Fig. 8. P8m. GFAP-IR in the central part of the retina. Fig. 9. P18m. Evenly distributed GFAP-IR Müller radial glia in the central retina. Figs. 10-12. rd/rd +/+ mouse retina. Scale bar 50 μm. Fig. 10. P14d. Central retina and (Fig. 11) peripheral retina. Note the very high density of GFAP-IR radial Müller glia. Fig. 12. P2m ONL has almost disappeared. Figs. 13-15. +/+ rd/rd mouse retina. Scale bar 50 μm. Fig. 13. P14d. Note the low numbers of GFAP-IR elements outside the OPL (cf. Figs 7, 10). Fig. 14. P2m Large numbers of GFAP-IR radial Müller glia penetrate the ONL (arrows). Fig. 15. P18m. Note the thick bundles of horizontal GFAP-IR fibers (arrows).
of cells was left in parts of the retina. The Müller cells gave rise to horizontal fibers that ran among what was left of the outer nuclear layer and the outermost cell rows of the inner nuclear layer. No outer plexiform layer could be identified. The overall density of GFAP-immunoreactivity did not appear higher than at P9m.

**+/+, rds/+ Mutant Mouse Retina**

In this mutant retina, heterozygous for the rds gene, degeneration proceeds slowly. Therefore, the earliest stage examined was P2m. Compared to the +/+, rds/rds at P2m, the outer nuclear layer of +/+, rds/+ showed a normal thickness (about ten rows of cell somata). Although the number of radial GFAP-IR glia was very high, fibrillar tangles and horizontal fibers were more scarce than in the homozygous +/+, rds/rds retina. P3m and P6m (Figs. 16, 17) were very similar in appearance to P2m.

At P16m the outer nuclear layer had decreased in thickness to approximately 3 to 4 cell rows in the central retina (Fig. 18). The density of GFAP-IR Müller cells had increased noticeably. In the peripheral retina, their distal processes penetrated the outer nuclear layer and covered its external surface. Large numbers of fibrillar tangles and horizontal elements were observed in the inner plexiform layer. The outer plexiform layer was heavily interspersed with immunoreactive elements.
**rd/rd rds/rds Mutant Mouse Retina**

In this mutant, the rate of photoreceptor cell loss was relatively slower than in the single homozygous rd retina. However, due to the action of the rds gene, remaining photoreceptor cells lacked outer segments. The density of GFAP-IR elements was intermediate to those of the single homozygous rd or rds retinae. At P11, small clusters of GFAP-IR Müller cells were observed, as well as immunoreactive astrocytes, especially in the vicinity of the optic disc. At P21 (Fig. 19), the labelling was comparable to that of the rds/rds mutant retina at P28.

**Discussion**

Müller radial glia accumulate glial fibrillary acidic protein in response to retinal injuries. This accumulation may occur as a response to experimental photoreceptor degeneration, lesions of the optic nerve and retina, or during inherited retinal dystrophy in the RCS rat. Thus, GFAP-accumulation seems to be a generalized response to several types of injuries. This observation raises questions about the changes in the retinal milieu that brings about this altered metabolism in Müller cells, and whether the GFAP accumulation in hereditary retinal disease is directly genetically regulated or an effect secondary to altered retinal functions. From our present observations on cats with inherited retinal dystrophy and on mice, homo- or heterozygous for the mutant genes rd and rds, we conclude the following:

1. GFAP-IR Müller cells are present in normal cat and mouse retinae.
2. There is a general increase with age in GFAP-IR radial Müller glia, both in terms of cell numbers and the extent of IR within induced cells as well as in other GFAP-IR elements in the retinae of cats and mice with hereditary retinal dystrophy. In early disease, the mouse mutant exhibits a greater GFAP-IR than the cat.
3. The increase in GFAP-accumulating Müller cells is not uniform across the retina. It is first observed in the periphery, and later in the central regions.
4. Comparing the different mutant mouse strains, there seems to be qualitative differences in the distribution of GFAP-IR elements. The numbers of horizontal elements and fibrillar tangles in the inner plexiform and nuclear layers are highest in the +/+ rds/rds retina, followed by the +/+ rds/+ and rd/rd rds/rds, whereas such elements appear to be more scarce in rd/rd rds/rd, followed by the rd/rd +/+ retinae.
5. Accumulation of GFAP-IR elements however occurs at the earliest stages in mice bearing the rd gene, ie, the rd/rd +/+ mouse, followed by the rd/rd rds/rds, the +/+ rds/rds, +/+ rds/+, and the normal mouse. This is in absolute agreement with the onset and time course of the degenerative changes.

Demonstration of GFAP-IR Müller cells in normal retinae, ie, retinae that have not undergone experimental or hereditary lesions, is not without precedence. Shaw and Weber observed GFAP-IR Müller cells in the retina of old albino rats. Since they could not observe any IR in nonalbino rats, they suggested that such immunoreactivity reflected age-related degeneration in the albino rat retina. Later, it was demonstrated that GFAP is present also in Müller cells of young albino rat retinae. However, significant species differences may exist for mammals, as is known to be the case among fish. In the rabbit retina GFAP immunoreactivity was found only in astrocytes of the optic axon layer.

From our observations it seems clear that although GFAP-immunoreactive Müller cells may be observed in varying numbers in normal retinae, their numbers increase dramatically as retinal degeneration progresses. Their presence in normal retinae may reflect naturally occurring cell death, at least in young animals. In the 12-week-old kitten, significant cell death in the course of retinal differentiation has been reported (Narfs, unpublished results).

Another aspect of this problem, that may be related to the species differences in the location of GFAP in the retina, as judged by immunocytochemistry is that, since GFAP and vimentin may form heteropolymer conjugates, antigenic epitopes may be differentially masked in different animal species. This could result in different locations of immunoreaction when different antibodies to GFAP are used. Thus, GFAP might coexist with other intermediate filaments in Müller cells of different animal species, but may be variably masked for immunocytochemical or immunocytochemical detection. It is, however, perhaps more probable that all Müller cells synthesize GFAP, and that its amount may vary in different species, or in healthy vs. pathologic retinae. In any case, we may conclude that elements expressing the epitope(s) recognized by the GFAP antibody used in the present study increase during the course of retinal degeneration.

GFAP-immunoreactive elements that are probably not related to the Müller cells also increase in number as degeneration progresses. Fibrillar tangles and horizontal fibers and fiber bundles in the inner plexiform and nuclear layers increase in numbers during degeneration. Preliminary double-labelling experiments with antibodies against vimentin fail to label elements other than the radial Müller glia, indicating
that the GFAP-IR fibrillar tangles and horizontal bundles originate in other cell types, perhaps astrocytes (Ekström and van Veen, unpublished observations). Whereas the fibrillar tangles and horizontal fibers seem preferentially associated with the rds gene as opposed to the rd gene (the dystrophic cat retina also contained numerous fiber bundles and tangles), horizontal GFAP-IR elements in the outer plexiform layer were observed in all retinae (except those in which this layer was not detectable owing to far-reaching degeneration).

GFAP immunoreactivity was invariably strong in the outer plexiform layer of the peripheral retina. It was not possible, using light microscopy, to identify these elements as lateral processes of the Müller cells. They might represent GFAP-IR horizontal cells. In some species of fish (but not all investigated) GFAP-immunoreactive horizontal cells have been detected. In mammals, however, GFAP-immunoreactive horizontal cells have so far not been described, although horizontal cells are rich in intermediate filaments.

The observation that GFAP accumulation invariably seems to proceed from the peripheral to the central retina requires comment. In both the retinal dystrophic cat, and the homo- and heterozygous rds mutant mouse, loss of photoreceptor cells proceeds from peripheral to central retina. On the contrary, in the rd mouse photoreceptor degeneration starts in the central retina and spreads towards the periphery. This is not reflected in the distribution of GFAP-accumulating elements. Thus, the accumulation of GFAP does not seem to be a local, direct effect of photoreceptor damage, but rather a generalized response, proceeding along similar patterns regardless of its cause. This conclusion is consistent with the observations by Shaw and Weber, who "found it to be a general rule that retinae which showed degenerative changes in any region expressed GFAP protein in every Müller fiber, irrespective of whether the Müller fiber was in a region of obvious degeneration or not."

The small numbers of clustered GFAP-IR Müller cells in the retinae of old normal mice (PI 8m) may simply reflect the difference between "normal" (age-related) retinal cell death, and cell death resulting from hereditary degenerative changes.

Further support may be sought from comparisons of the time course of photoreceptor damage and GFAP accumulation. In the cat, significant degenerative changes of photoreceptor cells were not observed until stage 2. We could show alterations already in the stage 0 (12-week-old kitten, homozygous for the defect but still with an ophthalmoscopically normal retina), in the form of increasing numbers of GFAP-immunoreactive Müller cells and other elements. In the mouse, heterozygous for the mutant gene rds (rds/+), degeneration of photoreceptor cells starts at the age of 2 months. At this age, numerous Müller cells were strongly immunoreactive for GFAP, although fibrillar bundles and tangles were rare. Already, 1 month later, such elements were abundant in spite of the slow course of degeneration in the (rds/+), mutant: the first indications of photoreceptor cell loss were discernible only at the age of 9–12 months. Since it has been suggested that the rds gene acts intracellularly within the photoreceptor cells, the early GFAP accumulation cannot be an effect secondary to photoreceptor cell death. Rather, it would seem that the abnormal GFAP expression results from generalized, genetically determined changes that precede the actual phenomenon of cell loss. Specifically, GFAP gene expression in Müller cells may be: (1) directly linked to the actions of the hereditary retinal degeneration genes; or (2) be an early reaction of Müller cells to altered photoreceptor cell metabolism, predicated by hereditary factors.

Key words: GFAP localization, retina, retinal degeneration, immunocytochemistry, rd mouse, rds mouse, Abyssinian cat

Acknowledgments

The authors are indebted to Miss Carina Rasmussen for excellent laboratory assistance and to Miss Inger Norling for photographic work, and to Mr. Henk Visser for the management of the mouse colony.

References

6. Schnitzer J and Karschin A: The shape and distribution of...


