Graefe's Arch Clin Exp Ophthalmol
(2001) 239:788–793

LABORATORY INVESTIGATION

DOI 10.1007/s004170100361

Timm Bredehorn
Matthias Clausen
Gernot Duncker
Renate Lüllmann-Rauch

Morphological and functional changes due to drug-induced lysosomal storage of sulphated glycosaminoglycans in the rat retina

Received: 7 February 2001
Revised: 6 August 2001
Accepted: 8 August 2001
Published online: 2 October 2001

Presented at the DOG Berlin 2000

T. Bredehorn (✉) · G. Duncker
Department of Ophthalmology,
University of Halle-Wittenberg,
Magdeburger Strasse 8,
06097 Halle-Saale, Germany
e-mail: timm.bredehorn@dso.de
Tel.: +49-345-5571881
Fax: +49-345-5571848

M. Clausen · R. Lüllmann-Rauch
Department of Anatomy,
University of Kiel, Kiel, Germany

Abstract A series of dicationic amphiphilic drugs, most of them immunomodulatory agents, are known to induce generalised lysosomal storage of sulphated glycosaminoglycans (GAGs) in rats and in cultured cells of several species including man. The present study deals with the cytological effects of two experimental immunomodulatory acridine derivatives upon the retina of rats. The animals were treated orally with compound CL-90.100 (3,6-bis[2-(diethylamino)ethoxy]acridine) or an analogue for periods up to 22 weeks at a dose range of 60–90 mg/kg body weight and the retinae examined by light and electron microcopy. ERG measurements were done initially and after 16 weeks of treatment. All types of retinal cells developed abnormal cytoplasmic vacuoles which represented the ultrastructural counterpart of lysosomal GAG storage as demonstrated by histochemical and cytochemical staining experiments. The retinal pigment epithelium and the Müller cells were most prominently affected, photoreceptor cells to a lesser degree, and retinal neurons to varying degrees. The topographical distribution of the drug as detected by fluorescence microscopy closely resembled the distribution of the GAG accumulation in the retinal layers. After treatment for 16 weeks, the a-and b-wave amplitudes in the ERG were significantly reduced compared with the controls. *Conclusion:* the glycosaminoglycan storage in pigment epithelium is reminiscent of that seen in some inherited mucopolysaccharidoses of humans. When a given cell type shows lysosomal accumulation of glycosaminoglycans as a consequence of impaired degradation, it can be assumed to be engaged in the turnover of glycosaminoglycans under normal conditions. Thus the present results suggest that not only the retinal pigment epithelium but also Müller cells, photoreceptor cells, and, to variable degree, retinal neurons are normally involved in the catabolism of sulphated glycosaminoglycans. We believe that the lysosomal storage of glycosaminoglycans caused secondary cellular disturbance responsible for the functional changes shown by electroretinography.

Introduction

Chronic administration of cationic amphiphilic drugs such as the antimalarial and anti-rheumatic drug chloroquine and the immunomodulatory drug tilorone are known to cause lipidosis and photoreceptor degeneration in the retina of humans and rats [3, 7, 14, 17,27]. On electroretinography, the chloroquine and tilorone retinopathies are characterised by decreasing b-wave and a-wave amplitudes [4, 7,13]. Two other cationic amphiphilic agents, the antiviral and immunomodulatory acridine derivatives, compounds CL-90.100 (3,6-bis[2-(diethyl-

amino) ethoxyl]acridine) and CL-246.738, have been reported to interfere with the lysosmal degradation of sulphated glycosaminoglycans (GAGs), thus inducing mucopolysaccharidosis in visceral organs of rats [2, 8, 9,11].

Since the retinal pigment epithelium and the neuroretina are considered to be engaged in the metabolism of GAGs of the interphotoreceptor matrix [15, 16,25], we were interested in determining whether or not the experimentally induced mucopolysaccharidosis affects the structure and function of the rat retina.

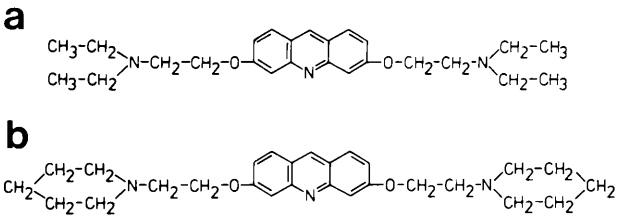


Fig. 1a, b Molecular structures of the experimental compounds (**a**) CL-90.100 and (**b**) CL-246.738. The only chemical difference is in the substituents on the nitrogen atoms of the side-chains (diethylamino versus piperidino)

Materials and methods

Materials

The acridine derivatives were a gift of the American Cyanamid Company, Lederle Laboratories (Pearl River, New York) and were used as trihydrochlorides. The molecular structures are shown in Fig.1.

Animal experiments

Female Wistar rats were used. At the beginning of the experiments they were 3–4 weeks of age and had body weights ranging between 75 and 90 g. They had free access to standard laboratory diet and tap water and lived at a light/dark rhythm of 12/12 h. The animal experiments were performed in accordance with the current version of the German Law on the Protection of Animals. The drugs were administered as additives to the ground chow at concentrations between 0.08% and 0.1%. The chow was offered in special containers which excluded food spillage. The body weights and food consumption were recorded every 3rd day and the resulting drug dosages were calculated. For either drug, the daily oral dosages ranged between 60 and 95 mg/kg body weight. The drug treatments were performed as long as compatible with the general physical condition of the animals. The body weight of the treated rats became reduced by 15% as compared to the controls.

In two independent series of experiments, nine rats were treated with compound CL-90.100 for 3 to 16 weeks and 17 rats received compound CL-246.738 for 5 to 22 weeks. Eight rats of the latter group were intended for ERG measurements. A total of 19 age-matched rats served as controls.

Electroretinographic (ERG) measurements

ERG measurements were performed before the start of drug treatment (eight rats, 16 eyes). Final measurement was done after 16 weeks of oral treatment in two rats (three eyes were measured; one eye could not be used because of problems with the anaesthesia). Six rats could not be used because of their poor condition or because they had died prior to measurement. Eleven age-matched rats (22 eyes) served as controls.

After 2 h of dark adaptation, the rats were anaesthetised under red light by intraperitoneal injection of a combination of 100mg/kg Ketavet ® (100mg/ml ketamine hydrochloride, Parke Davis) and 1.5 mg/kg Rompun ® (500mg xylazine hydrochloride, Bayer) [19].

ERG measurements were carried out with a new alternating current recording system [5]. The ERG was recorded at the surface of the cornea via a loop of platinum. The reference needle electrode, also of platinum, was inserted subcutaneously into the lower lid. The ground electrode was placed into the ear. A Grass PS 22 photostimulator emitted single flashes of 10 µs duration and 25% maximum intensity via an optic fibre 8 mm in diameter with an attached contact lens. At the surface of the cornea the flashes had an illuminance of 1.25 lumen/cm^2 [19].

Single records were used for calculating the ERG parameters. The a- and b-wave time to peak and the a-wave amplitude were measured from the light onset and the isoelectric line. The b-wave amplitude was measured from the negative peak of the a-wave [18]. The Mann-Whitney test was used for statistical analyses. Differences were considered statistically significant at $P<0.05$.

Morphological examination

One to three animals of either group were killed after drug treatments for 3, 6, 7, 8, 14, 15, 16 and 22 weeks, in parallel with age-matched controls. The animals were deeply anaesthetised with tribromoethanol (250 mg/kg, intraperitoneal injection) and killed by opening the left cardiac ventricle. Usually tissue fixation was achieved by transcardial vascular perfusion with glutaraldehyde (3% in 0.1 M phosphate buffer, pH 7.4). The ocular bulbi were removed and opened along the ora serrata. The retina was processed for ultrastructural examination according to standard methods. Semi-thin sections were stained with toluidine blue, ultra-thin sections were stained with uranyl acetate and lead citrate.

Fluorescence microscopy

The drugs under investigation are strongly fluorescent molecules. For studying the drug distribution in the retina, bulbi from drug-treated and control rats were snap-frozen in liquid nitrogen. Cryostat sections were freeze-dried [6], mounted with DePeX (Serva, Heidelberg) and viewed with a Zeiss Axiomat fluorescence microscope equipped with excitation filter BP 365/10 nm and barrier filter LP 395 nm.

Histochemical detection of GAGs

GAGs were visualised by staining with the cationic dyes cuprolinic blue and toluidine blue under conditions which allow electrostatic binding of the dyes selectively to polysulphated GAGs [20,21]: (1) cryostat sections (7 µm) of unfixed bulbi were stained for 6 h with 0.05% cuprolinic blue in 0.025 M sodium acetate buffer (pH 5.7) containing 0.3 M $MgCl_2$, (2) glutaraldehyde-fixed tissue samples were rapidly processed for embedding in glycolmethacrylate and sections (1 µm) were stained with 0.1% toluidine blue at pH 1.0.

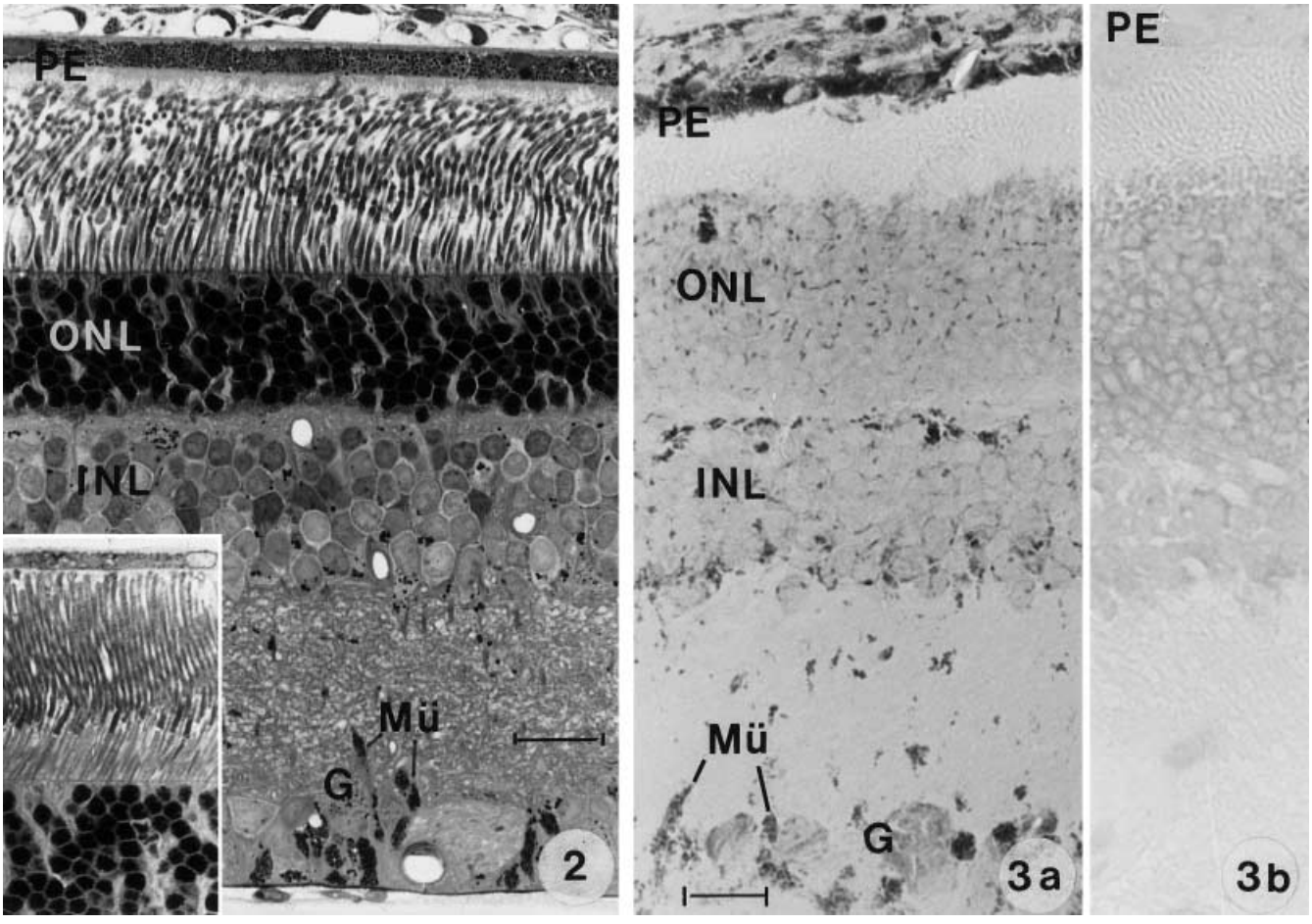


Fig. 2 Semi-thin sections of retinae of a rat treated for 14 weeks and of an age-matched control rat (*inset*). In the retina of the drug-treated rat abnormal cytoplasmic inclusions are seen in the pigment epithelium (*PE*), the outer and inner nuclear layers (*ONL*, *INL*), in the ganglion cell layer (*G*) and in Müller cells (*Mü*). In the pigment epithelium, the accumulation of abnormal inclusions leads to significant increase of the height of the cells (compare with the inset). Bar 20 µm

Fig. 3a, b Histochemical detection of sulphated GAGs in cryostat sections (7 µm). Retinae of a rat treated for 15 weeks (**a**) and a control rat (**b**). The sections were incubated with cuprolinic blue under conditions warranting selective staining of sulphated GAGs [21]. Positively reacting inclusions are seen in most cellular layers of the retina of the treated rat. (*Mü* Müller cell processes). In the control, such inclusions are absent. Bar 20 µm

Results

Morphological observations:

The two present acridine derivatives caused retinal alterations indistinguishable from each other. Therefore both groups of animals will be described collectively.

The overall structure of the retina usually resembled that of age-matched control retinae (Fig.2). Only in three of the rats treated with compound CL-90.100 (14–16 weeks), did the photoreceptors in the most peripheral part of the retina show alterations: the outer segments were shortened, distorted or missing and the inner segments were distorted (not shown). These alterations were not examined ultrastructurally. In the drug-treated rats, abnormal cytoplasmic inclusions could be seen in most retinal layers (Fig.2). In histochemical preparations incubated with cuprolinic blue (Fig.3) or toluidine blue, intensely staining inclusions were seen in the pigment epithelium, outer and inner nuclear layers, in Müller cells traversing the inner plexiform layer, in the ganglion cell layer and at the inner limiting membrane. These histochemical results indicated that the inclusions contained polysulphated GAGs [20]. The abundance of abnormal inclusions increased with the duration of drug treatment. In the retina of control rats such staining was absent (Fig.3). Examination by fluorescence microscopy revealed an uneven drug distribution throughout the retina (Fig.4): Drug-related fluorescence was seen in the same regions that displayed the storage of sulphated GAGs.

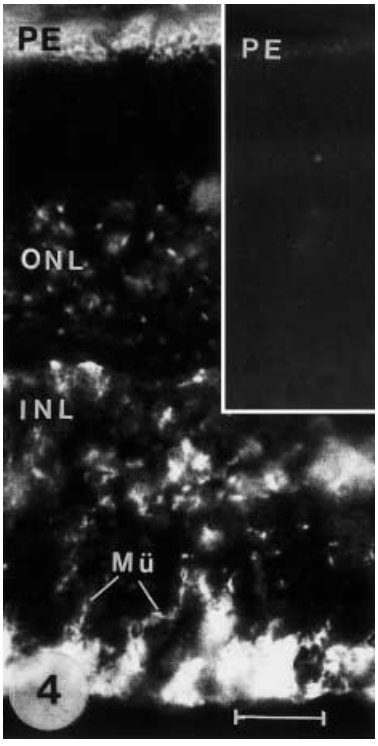


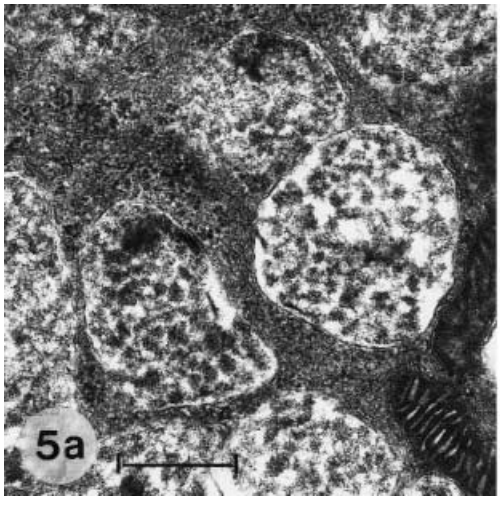
Fig. 4 Detection of the fluorescent compound CL-90.100 in the retina of a rat treated for 15 weeks. Freeze-dried cryostat sections viewed under the fluorescence microscope. The drug-related fluorescence shows a topographic distribution similar to that of the abnormal inclusions in Fig.3a. Retina of a control rat (*inset*). Only the pigment epithelium shows a very weak fluorescence. Abbreviations as in Fig.2 and Fig.3. Bar 20 µm

Electron microscopy

The abnormal cytoplasmic inclusions corresponded to membrane-limited organelles containing floccular material as shown for pigment epithelium (Fig.5) and a photoreceptor perikaryon (Fig.6). In the pigment epithelium, occasionally abnormal storage material was seen to be contained along with a freshly engulfed outer segment fragment within in the same organelle (Fig.5); such observations suggest that the storage lysosomes could fuse with newly formed phagosomes. The overall frequency of outer segment phagosomes was similar to that in controls, suggesting that the digestion of engulfed photoreceptor membranes was not impaired. In Müller cells, the storage lysosomes were particularly numerous in the cell bodies, in the radial pillars traversing the inner plexiform layer and in the basal end feet adjacent to the inner limiting membrane. In photoreceptor cells, the storage organelles were usually located in the outward-orientated pole of the perikaryon. In retinal ganglion cells and neurons of the inner nuclear layer the occurrence of inclusions was variable.

Electroretinographic findings

Initial ERG parameters of the animals designated for treatment and the controls were in the normal ranges. Obvious changes in ERG parameters were found after treatment. A reduction of b- and a-wave amplitudes was seen after chronic treatment with compound CL – 246.738 (Fig.7). After treatment for 16 weeks, the



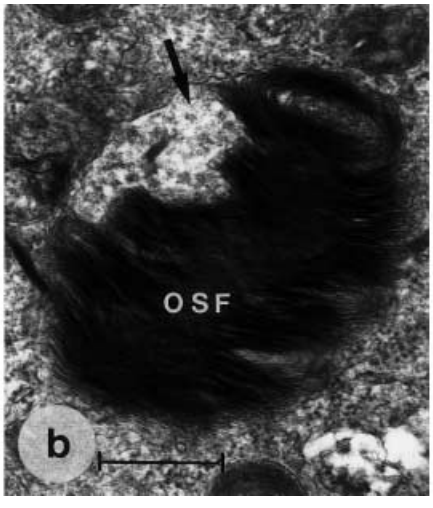


Fig. 5a, b Abnormal inclusions in the pigment epithelium of rats treated for 14 weeks. (**a**) Membrane-limited organelles are seen containing floccular material of relatively low electron densitiy. (**b**) Freshly engulfed outer segment fragment (*OSF*). The outer segment discs are contained within the same organelle as the drug-induced storage material (*arrow*). Bars 0.5 µm

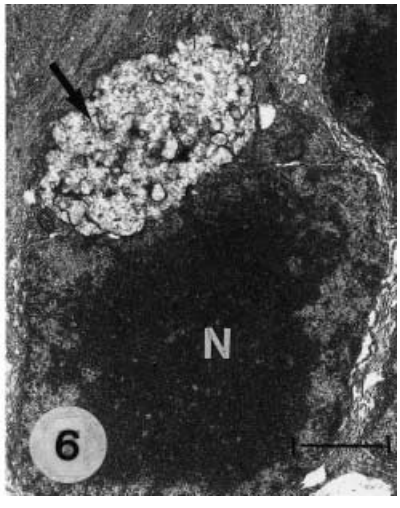


Fig. 6 Abnormal inclusion (*arrow*) in a photoreceptor perikaryon of a rat treated for 16 weeks. The process towards the inner segment is orientated to the top of the picture. (*N* nucleus of the photoreceptor cell). Bar 1 µm

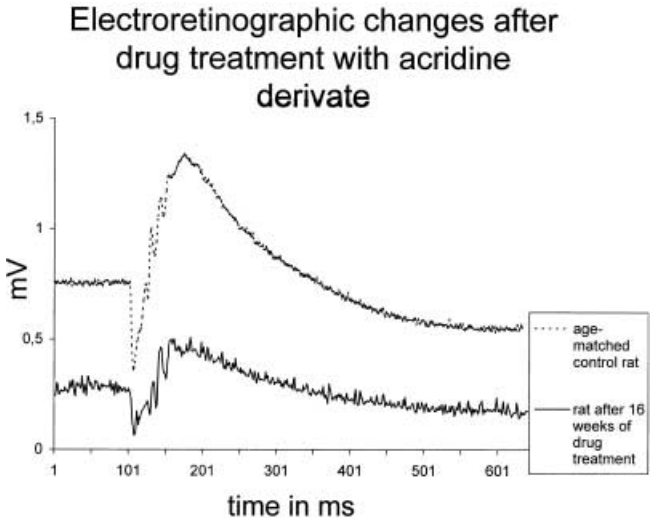


Fig. 7 Single electroretinographic recordings showing characteristic reduction of the a- and b-wave parameters of a rat after treatment for 16 weeks with compound CL 246.738 (*solid line*) compared to an age-matched control rat (*dashed line*). The rats were dark adapted for 2 h. Flash duration was 10µs and flash intensity was 1.25 lumen/cm^2

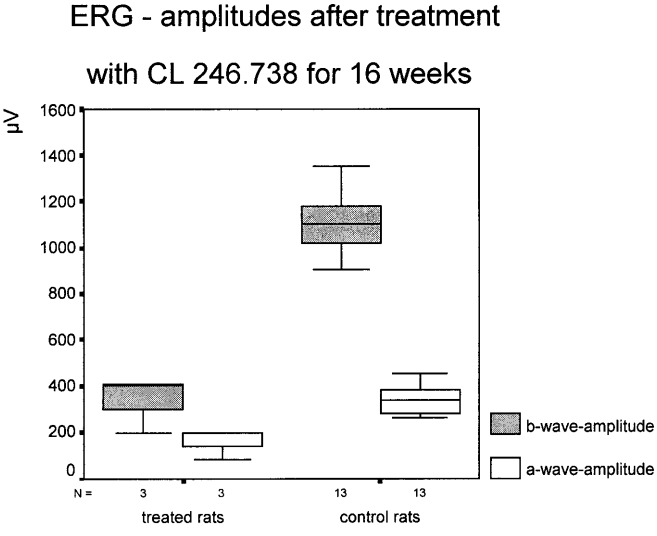


Fig. 8 The effect of chronic drug administration on the a- and b-wave amplitudes. Box plots with median, minimum, maximum, 0.25 quartile and 0.75 quartile of the a- and b-wave amplitudes in three eyes from rats after 16 weeks of treatment with compound CL-246.738 (60–95 mg/kg body weight, orally) compared to 13 eyes from rats of the age-matched controls. Stimulus intensity was 1.25 lumen/cm2

a-wave amplitude in three eyes from two rats decreased to a median of 195 µV compared with the median of 340 µV in 13 eyes from seven rats of the age matched controls (P<0.05) (Fig.8). B-wave amplitude was reduced in three eyes from two rats to a median of 400 µV after treatment for 16 weeks compared with the median of 1100 µV in 13 eyes from seven rats of the age matched controls (P<0.05)(Fig.8). The values after treatment for 16 weeks for the a- and b-wave time to peak compared with the age-matched controls showed no significant difference (P>0.05).

Discussion

The present results show that the retina of rats participates in the mucopolysaccharidosis induced by acridine derivatives and the retinal function is impaired. While the present study did not include the quantitation of GAGs, results from other organs may be quoted. In the liver and spleen of rats treated with the same acridine derivatives as the present animals, the GAGs were increased by factors of approximately 50 and 20, respectively [9]. The drug-induced GAG storage was not evenly distributed throughout the retina. The pigment epithelium and the Müller cells were most prominently affected. Furthermore, many photoreceptor perikarya showed lysosomal storage. In the neurons the GAG storage was moderate or weak. The heterogeneous distribution of GAG storage may be due to cell type-specific differences in the turnover of GAGs. A similar heterogeneity is known for the inherited mucopolysaccharidoses, which on the ultrastructural level are associated with cytoplasmic vacuoles similar to the present ones [1, 12, 22, 23, 24, 26].

The reduction of b-wave amplitudes has been attributed to changes in the neuroretina, particularly in Müller cells and the inner nuclear layer [7,10]. Mucopolysaccharidosis-like changes caused by chronic treatment with acridine derivatives were found in the Müller cells and to a lesser degree the inner nuclear layer. These changes are considered responsible for the reduction of b-wave amplitudes. The engagement of retinal pigment epithelium and neuroretina in the metabolism of GAGs contained in the interphotoreceptor matrix may be somehow related to the a-wave amplitude reduction as seen in the drug-treated rats. We asssume that the storage of GAGs caused secondary cellular disturbance which are responsible for the functional changes shown by electroretinography. The mechanisms linking the lysosomal overload and the cellular dysfunctions are unknown at present. We would recommend regular ocular monitoring in humans if acridine derivatives come into clinical use.

Acknowledgements The excellent technical assistance of Dagmar Niemeier and Sonja Seiter is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft (Grant No. Lu 172).

References

1. Aguirre G, Stramm L, Haskins M (1983) Feline mucopolysaccharidosis VI: general ocular and pigment epithelial pathology. Invest Ophthalmol Vis Sci 24: 991–1007
2. Angier RB, Citarella RV, Damiani M, Fabio PF, Fields TL, Kang SM, Lin Y, Lindh HF, Murdock KC, Petty SR, Wilkinson RG, Lang SA (1983) Synthesis of 3,6-bis(aminoalkoxy)acridines and their effect on the immune system. J Med Chem 26: 1710–1715
3. Bernstein HN, Ginsberg J (1964) The pathology of chloroquine retinopathy. Arch Ophthal 71: 238–245
4. Bredehorn T, Duncker G (2000) Tiloron-induzierte funktionelle Veränderungen der Rattenretina. Klin Monatsbl Augenheilkd 216: 219–222
5. Bredehorn T, Duncker G, Kuper H, Papst N (1994) A new system for recording electroretinographical potentials in animals. Ophthalmologica 208: 98–104
6. Csillik B, Kalman G (1967) Vacuumless freeze-drying: its application in catecholamine chemistry. Histochemie 7: 275–280
7. Duncker G, Schmiederer M, Bredehorn T (1995) Chloroquine-induced lipidosis in rat retina: a functional and morphological study. Ophthalmologica 209: 79–83
8. Fischer J (1996) Drug-induced glycosaminoglycan storage: dose-dependent changes in the pattern of accumulated glycosaminoglycans in cultured bovine and human fibroblasts. Biochem Pharmacol 52: 1331–1337
9. Grave S, Lüllmann H, Lüllmann-Rauch R, Osterkamp G, Prokopek M (1992) Induction of mucopolysaccharidosis in rats by treatment with immunostimulatory acridine derivatives. Toxicol Appl Pharmacol 114: 215–224
10. Gouras P (1970) Electroretinography: some basic principles. Invest Ophthalmol 9: 557–569
11. Handrock K, Laschke A, Lüllmann-Rauch R, Vogt RD, Ziegenhagen M (1992) Lysosomal storage of sulfated glycosaminoglycans in cultured fibroblasts exposed to immunostimulatory acridine derivatives. Toxicol Appl Pharmacol 114: 204–214
12. Haskins ME, Aguirre GD, Jezyk PF, Desnik RJ, Patterson DF (1983) The pathology of the feline model of mucopolysaccharidosis I. Am J Pathol 112: 27–36
13. Hennekes R (1989) Klinische Elektroretinographie. Fortschr Ophthal 86: 146–150
14. Ivanina TA, Zueva MV, Lebedeva MN, Bogoslovsky AI, Bunin AJ (1983) Ultrastructural alterations in rat and cat retina and pigment epithelium induced by chloroquine. Graefes Arch Clin Exp Ophthalmol 220: 32–38
15. Landers RA, Hollyfield JG (1992) Proteoglycans in the mouse interphotoreceptor matrix. VI. Evidence for photoreceptor synthesis of chondroitin sulfate proteoglycan using genetically fractionated retinas. Exp Eye Res 55: 345–356
16. Landers RA, Tawara A, Varner HH, Hollyfield JG (1991) Proteoglycans in the mouse interphotoreceptor matrix. VI. Retinal synthesis of chondroitin sulfate proteoglycan. Exp Eye Res 52: 65–74
17. Lüllmann-Rauch R, Lüpkes S (1987) Drug-induced degeneration of photoreceptor cells. Adv Biosci 62: 471–476
18. Marmor MF, Arden GB, Nilsson SFG, Zrenner E (1989) Standard for clinical electroretinography. Arch Ophthalmol 107: 816–819
19. Schaeppi U, Krinke G, Fink X, Hofer R, Duennenberger D (1988) Electroretinography in rats. Agents Actions 24: 395–402
20. Scott JE, Dorling J (1965) Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. Histochemie 5: 221–233
21. Scott JE, Orford CR, Hughes EW (1981) Proteoglycan-collagen arrangements in developing rat tail tendon. Biochem J 195: 573–581
22. Shull RM, Helman RG, Spellacy E, Constantopouls G, Munger RJ, Neufeld EF (1984) Morphologic and biochemical studies of canine mucopolysaccharidosis I. Am J Pathol 114: 487–495
23. Stramm LE, Haskins ME, Aguirre GD (1989) Retinal pigment epithelial glycosaminoglycan metabolism: intracellular versus extracellular pathways. Invest Ophthalmol Vis Sci 30: 2118–2131
24. Stramm LE, Wolfe JH, Schuchman EH, Haskins ME, Patterson DF, Aguirre GD (1990) ß-Glucuronidase mediated pathway essential for retinal pigment epithelial degradation of glycosaminoglycans. Disease expression and in vitro disease correction using retroviral mediated cDNA transfer. Exp Eye Res 50: 521–532
25. Tawara A, Varner HH, Hollyfield JG (1989) Proteoglycans in the mouse interphotoreceptor matrix. II. Origin and developement of proteoglycans. Exp Eye Res 48: 815–839
26. Vogler C, Birkenmeier EH, Sly WS, Levy B, Pegors C, Kyle JW, Beamer WG (1990) A murine model of mucopolysaccharidosis VII. Am J Pathol 136: 207–217
27. Wetterholm DH. Winter FC f(1964) Histopathology of chloroquine retinal toxicity. Arch Ophthal 71: 82–87