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Colocalization of retinal dystrophin and actin in postsynaptic dendrites of rod and cone photoreceptor synapses

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Abstract. In this paper we demonstrate immunostaining specific for dystrophin in photoreceptor synapses of human, bovine and rat retinas. Cryosections of retinas incubated with dystrophin-specific monoclonal antibodies displayed a punctuate staining pattern in the outer plexiform layer. This pattern resulted from binding of the antibodies to synaptic complexes of both rods and cones, shown by double-labelling with antibodies to either synaptophysin or actin. Confocal laser fluorescence microscopy demonstrated that dystrophin staining colocalized predominantly with actin, which is concentrated in the postsynaptic portions of the synaptic complex. No significant dystrophin immunolabel was seen in the presynaptic terminals labelled with antibodies to synaptophysin, a marker of synaptic vesicles. Immunoblot analysis confirmed the presence of $\approx$420 kDa and $\approx$360 kDa dystrophin-like polypeptide bands associated with membranes of the bovine retina. We speculate that retinal dystrophin is involved in the linkage of actin filaments to the postsynaptic plasma membrane. Such a linkage may be important for the generation of synaptic microdomains and for certain phenomena of synaptic plasticity. The absence of dystrophin in patients suffering from Duchenne's muscular dystrophy is accompanied by visual problems and abnormalities of the electroretinogram. Therefore it is likely that retinal dystrophin plays a role in certain stages of synaptic transmission between photoreceptors and the postsynaptic dendritic complex formed by horizontal and bipolar cells.

Introduction

Duchenne's muscular dystrophy (DMD) is a X chromosome-linked, progressive disease of the muscular system, which occurs in boys with an incidence of $\approx 1:3500$ and leads to death usually before the age of 20 years (Moser 1984). The molecular basis of this disease is the absence of the DMD gene product. The DMD gene codes for the protein dystrophin, a rod-shaped homodimeric molecule with 3685 amino acid residues and a calculated molecular weight of 427 000 (Koenig et al. 1987a, b; for review see Mandel 1989; Anderson and Kunkel 1992; Ahn and Kunkel 1993).

Dystrophin is a peripheral membrane protein that folds into four distinct structural domains. The amino-terminal domain of dystrophin shows sequence homologies to the amino-terminal, actin-binding domains of α-actinin and β-spectrin. The largest part of dystrophin is composed of a rod-shaped portion with 24 repeat regions that share structural similarities with the rod domains of spectrin and α-actinin. The third, cysteine-rich domain resembles the carboxy-terminus of α-actinin from *Dictyostelium discoideum*. The fourth domain of dystrophin comprises the short carboxy-terminal region, which shows partial homology to the recently discovered dystrophin-related proteins (Love et al. 1989). This domain anchors dystrophin to a complex of membrane proteins of which one appears to bind extracellular matrix components, such as laminin or fibronectin (Ervasti and Campbell 1993; Lakonishok et al. 1992).

In skeletal muscle fibres dystrophin is attached to the inner surface of the plasma membrane. Dystrophin appears to be important for stabilization of the plasma membrane (Menke and Jokusch 1991) and for the transmission of intracellular forces to the extracellular matrix (for review see Brown and Lucy 1993). In addition to skeletal muscle, dystrophin has been also shown to occur, albeit less abundantly, in cardiac muscle, smooth muscle and nervous tissue (Lidov et al. 1990; Byers et al. 1991). In brain, dystrophin occurs as a $\approx$420 kDa spliced variant that appears to be localized mainly postsynaptically (Lido et al. 1990, Ahn and Kunkel 1993). Intellectual deficiencies frequently observed in patients carrying DMD were tentatively explained by the absence of dystrophin from cortical synapses. Recently two abstracts were published (Pillers et al. 1992, 1993) indicating that DMD patients suffer from certain visual problems that are most pronounced during dark-adaptation. Since dystrophin-like immunofluorescence has been observed in the outer plexiform layer of the mouse retina (Zhao et al. 1991), we became interested to look for dystrophin in the retinas of other species including men, and to determine its cellular and subcellular location.

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Materials and methods

Immunocytochemistry

Retinas were processed for immunocytochemistry by shock-freezing in liquid nitrogen-cooled isopentane. Cryostat sections 5 µm in thickness were cut with a Frigocut 2800 E (Reichert-Jung, Nussloch, Germany). Human retinas were obtained from a male person 1 h post mortem. Bovine eyes from animals of both sexes were obtained from a local slaughterhouse; retinas were isolated from the eyes within 30 min after death. Retinas from adult Wistar rats of both sexes were prepared within 10 min post mortem. Cryosections were thawed on poly-L-lysine-coated coverslides and dried on a hot plate (37° C) for 1 h.

For immunostaining two different dystrophin-specific monoclonal antibodies were used, which were specific for peptide sequences from the carboxy-terminus and also from the central rod domain ("Dys2" and "Dys1", Medac, Hamburg, Germany). The sequences against which these antibodies have been raised are specific for dystrophin and do not show significant sequence homology to dystrophin-related proteins (Hoffman et al. 1987; Nicholson et al. 1989; Ohlendieck et al. 1991; Bulman et al. 1991; Zubrzycka-Gaarn et al. 1991) or to the recently sequenced postsynaptic 87K protein (Wagner et al. 1993). The antibodies react with dystrophin in normal mice but not in mdx mice (Bulfield et al. 1984), which do not contain dystrophin but do contain dystrophin-related proteins. The monoclonal antibodies were used overnight at 4° C at a 1:5 dilution in phosphate-buffered saline (PBS), pH 7.4. The binding of the monoclonal primary antibodies was visualized with goat anti-mouse IgG as secondary antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma, Deisenhofen, Germany) at a 1:30 dilution in PBS, pH 7.4 (1 h, 22° C). Sections were mounted in 60% glycerol in PBS, pH 7.4, containing 1.5% *n*-propyl gallate (Merck, Darmstadt, Germany) to reduce photobleaching of the samples.

Presynaptic photoreceptor terminals were labelled with a polyclonal antibody against synaptophysin (Wiedenmann and Franke 1985) as previously described (Schmitz and Drenckhahn 1993). The antibody was a kind gift of Dr. B. Wiedenmann (Universität Steglitz, Berlin). The binding of this antibody was detected with goat anti-rabbit IgG as secondary antibodies coupled to tetramethyl rhodamine (TRITC) in a 1:30 dilution in PBS, pH 7.4 (1 h incubation time at 22° C). The postsynaptic dendritic complex of both rod and cone photoreceptor synapses was visualized with a polyclonal antibody against actin as previously described (Schmitz and Drenckhahn 1993). The binding of this primary antibody was visualized with goat anti-rabbit IgG labelled with TRITC (1:30 dilution, 1 h incubation at 22° C).

For double labelling, sections were sequentially incubated first with the monoclonal Dys2 antibodies and goat anti-mouse IgG conjugated to FITC at the concentrations indicated above and then with the polyclonal actin antibodies and goat anti-rabbit IgG conjugated to TRITC also at the concentrations indicated above. Controls were performed by omitting the primary antibodies. Sections were analysed either with a conventional fluorescence microscope (Olympus BH2, equipped with an epifluorescence optical system) or with a confocal laser scanning microscope (MRC 600; Biorad, Munich, Germany) attached to an Olympus BH2 microscope and equipped with a dual-channel scanning option for TRITC- and FITC-fluorescence.

Preparation of retinal membranes

Bovine retinas were disrupted using an Ultraturrax (type TP 18/10; Janke and Kunkel GmbH, Staufen, Germany) in buffer containing 15 m*M* Na_2HPO_4, pH 7.4, 1 m*M* ethylene glycol bis(2-aminoethyl)-tetraacetic acid (EGTA), 1 m*M* $MgCl_2$ and 1 m*M* phenylmethylsulphonyl fluoride (PMSF) for 3 min at 4° C. For preparation of crude membranes, the homogenate was overlayered on a cushion containing 50% sucrose (w/v) in 15 m*M* Na_2HPO_4 (pH 7.4), 1 m*M* EGTA, 1 m*M* $MgCl_2$, 1 m*M* PMSF, and centrifuged for 50 min at 20 000 *g* at 4° C. The pellet was discarded and the supernatant containing the retinal membranes was used for immunoblotting. Protein concentrations were determined according to Bradford (1976).

Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Retinal membrane proteins (100 µg per lane) were separated electrophoretically on a linear 2.5–10% polyacrylamide gradient gel (Laemmli 1970). Proteins were electrotransferred to nitrocellulose for 6 h at 50 V and 150 mA according to Towbin et al. (1979). After blocking unspecific binding sites with 5% skimmed milk in PBS (pH 7.4) for 30 min at 22° C, nitrocellulose strips were incubated overnight at 4° C with monoclonal Dys2 antibodies at a 1:5 dilution in PBS (pH 7.4). The binding of the primary antibodies was visualized with goat anti-mouse antibodies conjugated to horseradish peroxidase (Biorad, Richmond, Va., USA). α-Chloronaphthol (Biorad) was used as a chromogen for visualization of peroxidase activity. As high molecular weight markers we used α,β-spectrin of pig erythrocytes (M_r 240 000 and 220 000, respectively) and also dynein heavy chain of chick brain (M_r 440 000). These marker proteins were detected in pig erythrocyte ghosts by antibodies to spectrin (Drenckhahn et al. 1991) and in chick brain extracts by antibodies to dynein heavy chain (Steuer et al. 1990; antibody obtained from Sigma).

Results

Immunoblotting of bovine retinal membranes with antibodies specifically directed against dystrophin (Dys2) revealed that dystrophin is present in the retina (Fig. 1). A polypeptide band was detected with the typical molecular weight (≈420 kDa) of dystrophin. In addition, another protein band at approx. 360 kDa was labelled, which may represent a degradation product or a retinal splice variant of dystrophin. Degradation products of dystrophin are very frequently observed since dystrophin is extremely sensitive to proteolytic degradation (Arahata et al. 1988, 1989). When human and rat retinas were used at 6 h post mortem, dystrophin was no longer detectable (unpublished data).

The cellular and subcellular location of dystrophin was determined by immunofluorescence microscopy us

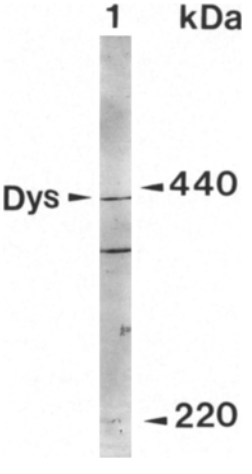

Fig. 1. Immunoblot analysis of bovine retinal membrane proteins. *Lane 1*, 100 µg of membrane proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose and immunostained for dystrophin with the monoclonal Dys2 antibody. A polypeptide band at approx. 420 kDa, the typical molecular weight of dystrophin, was recognized. Another polypeptide band at 360 kDa may represent a degradation product or a retinal splice variant of dystrophin

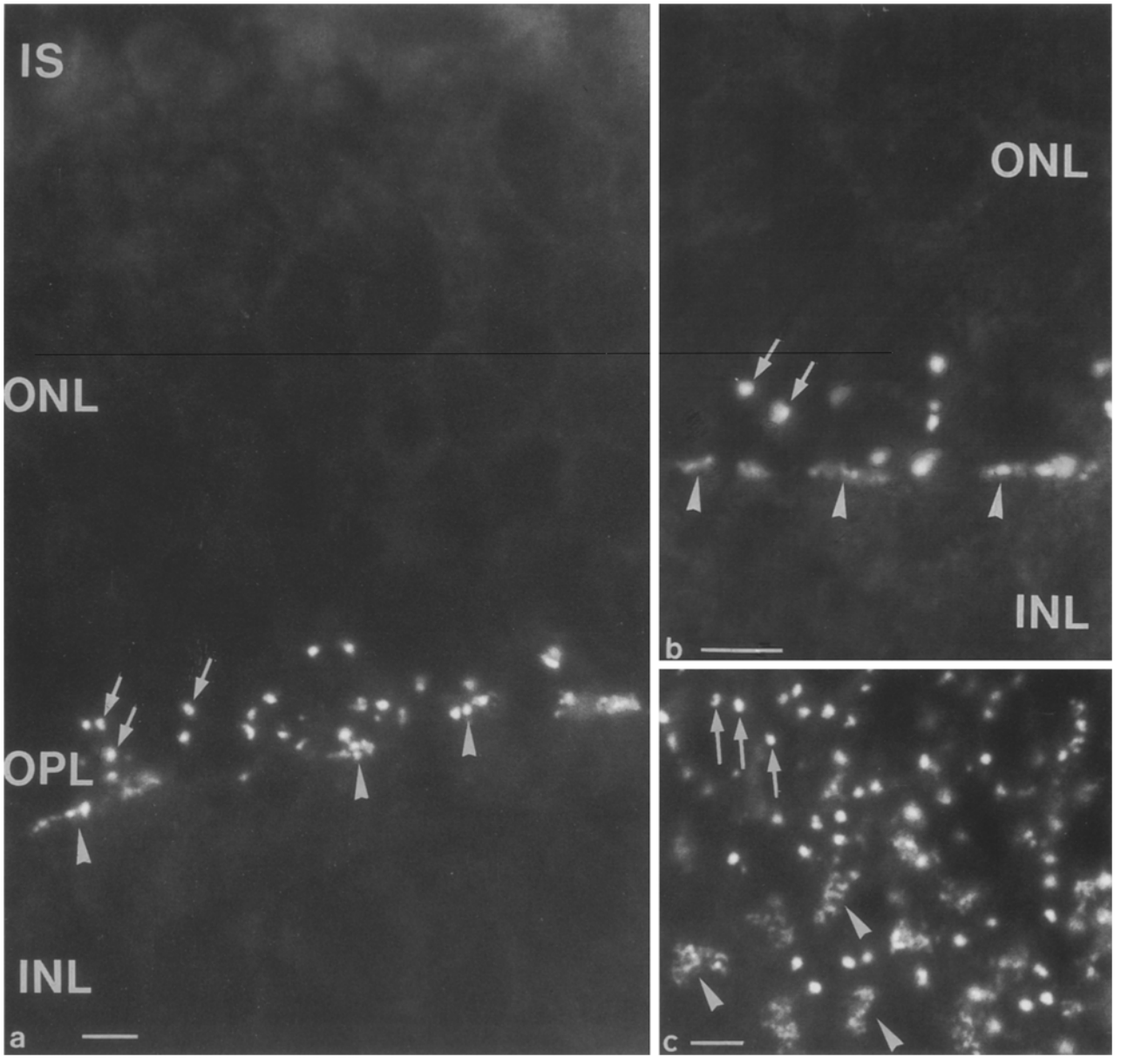

Fig. 2a–c. Distribution of dystrophin in cryostat sections of the human retina labelled with monoclonal antibodies to dystrophin (Dys2). The sections were analysed with a conventional fluorescence microscope. **a, b** Cross-sections of the retina: note the strong dystrophin immunoreactivity in the outer plexiform layer (*OPL*). Two immunofluorescent structures could be discriminated: single, dot-like immunofluorescent structures in the outer zone of the OPL (*arrows*), and streak-like ones in the inner zone of the OPL (*arrowheads*). The streak-like immunofluorescent structures in the inner zone of the OPL are discontinuous and consist of clusters of smaller subunits (*arrowheads*). Tangential section through the OPL. *ONL,* Outer nuclear layer; *INL,* inner nuclear layer; *IS,* inner segments. *Scale bars,* 5 μm

ing tissue sections of the human, bovine and rat retina. Both the human and bovine retinas are mixed retinas containing both rod and cone photoreceptors. In contrast to these species the rat retina virtually contains only rod photoreceptors and lacks cone photoreceptors (for review see Kolmer 1936; Hogan et al. 1971; Stell 1972). Immunolabelling of the human retina with monoclonal antibodies to dystrophin (Dys2) resulted in a bright label of the outer plexiform layer (Fig. 2). Within the outer plexiform layer of the human retina two different immunofluorescent structures could be discriminated. In the outer zone of the outer plexiform layer (close to the outer nuclear layer) the immunolabelled structures were dot-like and displayed a uniform size of approx. 1 μm in diameter (Fig. 2). In the inner zone of the outer plexiform layer (located closer to the inner nuclear layer) larger, streak-like immunolabelled structures with a length of approx. 3–5 μm were observed (Fig. 2). These larger immunostained structures in the inner zone of the OPL resulted from the labelling of aggregates of smaller subunits (Fig. 2c).

In the bovine retina a similar distribution of dystro-

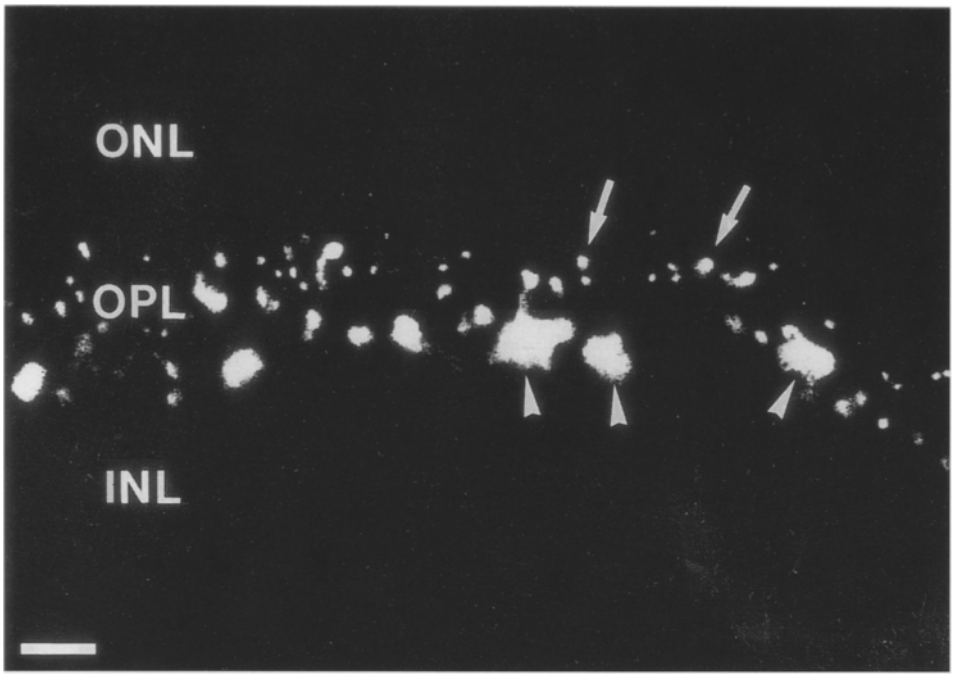

Fig. 3. Localization of dystrophin in cryostat cross-sections of the bovine retina examined with the laser scanning microscope. Similar to the human retina, the bovine retina displays two different immunofluorescent dystrophin signals in the OPL: dot-like dystrophin signals in the outer zone of the outer plexiform layer (*arrows*) and larger dystrophin signals in the inner zone of the outer plexiform layer (*arrowheads*). For abbreviations see legend to Fig. 2. *Scale bar,* 5 μm

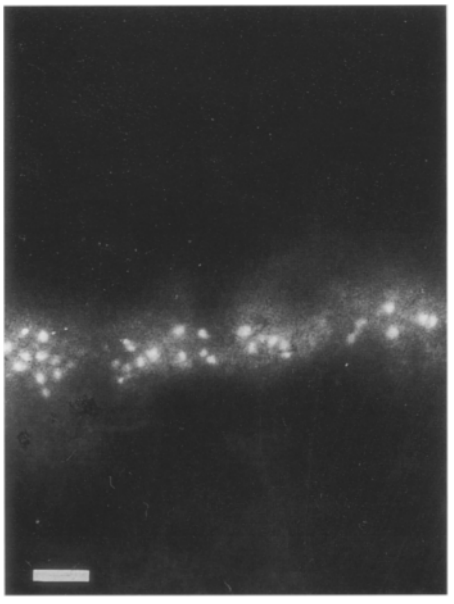

Fig. 4. Transverse cryostat section through the OPL of the all-rod retina of the rat stained with antibodies to dystrophin and examined with a conventional fluorescence microscope. Similar to the human and bovine retinas, the rat retina shows a strong antibody labelling of the OPL. In contrast to the rod- and cone-containing human (cf. Fig. 2) and bovine (cf. Fig. 3) retinas the all-rod retina of the rat only displayed small, dot-like immunolabelled structures in the OPL (approx. 1 μm in diameter). For abbreviations see legend to Fig. 2. *Scale bar,* 5 μm

phin was observed (Fig. 3). The outer plexiform layer displayed small dot-like dystrophin-containing structures in the outer zone and larger dystrophin-containing structures in the inner zone. An identical but weaker staining pattern was obtained with another dystrophin-specific monoclonal antibody, Dys1 (data not shown). We also localized dystrophin in the rat retina where cones are virtually absent (Fig. 4). In the outer plexiform layer of the rat retina, the antibodies to dystrophin labelled small dot-like structures of approx. the same size as the small dots seen in the bovine and human retinas. In contrast to the findings on the human and bovine retinas, larger fluorescent structures were not observed in the outer plexiform layer of the rat retina, supporting our view that the large immunolabelled structures in the human and bovine retina represent synaptic complexes of cones.

The typical size and distribution of the dystrophin signals in the outer plexiform layer of the retina from the described species suggested an association of dystrophin with rod and cone photoreceptor synapses. In order to verify the synaptic localization of dystrophin, we performed double-labelling studies with antibodies to dystrophin and synaptophysin. Synaptophysin is a membrane protein of synaptic vesicles (Wiedenmann and Franke 1985) including those of photoreceptor presynaptic terminals (Mandell et al. 1990). As a marker of the postsynaptic dendritic complexes we used a polyclonal antibody to actin (Schmitz and Drenckhahn 1993). As shown in Fig. 5, the dystrophin-positive structures were located within the actin-containing postsynaptic complexes. Double-labelling with anti-synaptophysin revealed that the majority of the dystrophin-containing structures was located close to the presynaptic photoreceptor terminals but did only rarely overlap with them (Fig. 5).

Discussion

In this paper we demonstrate dystrophin-like immunostaining in the outer plexiform synaptic layer of human, bovine and rat retinas. Double-labelling experiments us-

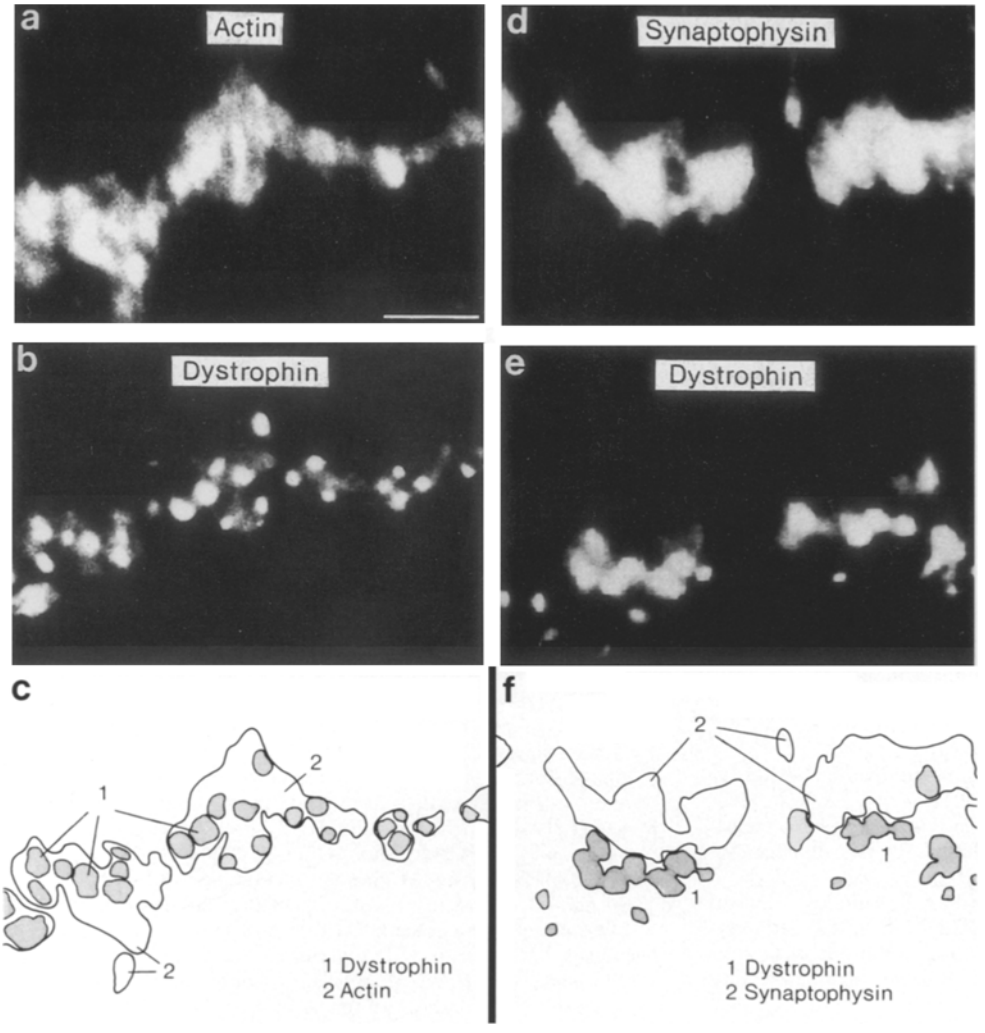

Fig. 5. Double-labelling of bovine retinas with antibodies against **b, e** dystrophin Dys2, **a** actin (as postsynaptic marker) and **d** synaptophysin (as marker of the presynaptic terminals). The dystrophin label was superimposed on the actin label in **c** and on the synaptophysin label in **f**. Note that the dystrophin label is located within the actin label (**a–c**). The synaptophysin and dystrophin signals (**d–f**) do not colocalize but are located complementary to each other. Micrographs obtained with laser scanning microscope. *Scale bar*, 5 μm

ing antibodies against synaptophysin and actin as pre- and postsynaptic markers of photoreceptor synapses (Schmitz and Drenckhahn 1993) indicate that the dystrophin immunolabel is confined to the photoreceptor synaptic complexes. Due to the co-localization of the dystrophin and actin immunolabels we conclude that retinal dystrophin is predominantly associated with the postsynaptic dendritic complexes of bipolar and horizontal cells. This conclusion is further supported by our observation that the dystrophin signal was located close to the synaptophysin-labelled photoreceptor terminals, but did not significantly overlap with them. In the rod- and cone-containing bovine and human retinas, the dystrophin-labelled structures (synapses) displayed two different sizes, i.e. dot-like structures in the outer zone and larger, streak-like structures in the inner zone of the outer plexiform layer. Since the size and distribution of the dystrophin label strongly resembled the pattern of rod and cone synapses (for review see Hogan et al. 1971; Stell 1972; Rodieck 1973; Dowling 1987) we suggest that retinal dystrophin is present in both the small synaptic complexes of rods and the larger synaptic complexes of cones.

The functional importance of dystrophin in the retina can be extrapolated from visual deficiencies diagnosed in patients suffering from Duchenne's muscular dystrophy (DMD). These visual problems and abnormalties of the electroretinogram (summarized as "Oregon Eye Disease"; Pillers et al. 1992, 1993) are most likely the result of the absence of dystrophin from photoreceptor synapses in these patients. These findings raise the question concerning the role of dystrophin in photoreceptor synapses. In a previous study we could show that the postsynaptic dendritic complex of bipolar and horizontal cells is particularly rich in actin filaments (Schmitz and Drenckhahn 1993). Since dystrophin is an actin-binding protein and appears to link integral membrane proteins to the actin cytoskeleton, it is possible that dystrophin may serve to immobilize functionally important membrane proteins at the postsynaptic membrane of photoreceptor synapses. Such membrane proteins may comprise transmitter receptors, ion channels and other components important for synaptic transmission.

Dystrophin-mediated connections of the actin cytoskeleton to the plasma membrane may also be important for shape changes and rearrangements of the postsynap-

tic dendritic complex. Such morphological changes have been mainly observed in the photoreceptor synapses of non-mammalian species (Schaeffer and Raviola 1975, 1978; Wagner 1980; Schmitz and Drenckhahn 1991), but it is conceivable that this phenomenon of synaptic plasticity also occurs in the photoreceptor complex of mammalian species.

In conclusion we can make the following statement: retinal dystrophin is concentrated in photoreceptor synapses of the mammalian retina most likely within the postsynaptic complex. Since the absence of dystrophin in DMD patients is accompanied by visual problems, it is likely that retinal dystrophin plays a role in certain stages of synaptic transmission between photoreceptors and the postsynaptic dendritic complex that is formed by horizontal and bipolar cells. Further clues to the function of retinal dystrophin may come from immunoelectron microscopic studies and from studies on the ultrastructure of photoreceptor synapses in DMD patients.

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Note added in proof. After submission of this manuscript a study of Pillers et al. (1993) appeared in Nature Genet 4:82–86 in which different splice variants of dystrophin were demonstrated in the human retina.

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