 Human Molecular Genetics, 1997, Vol. 6, No. 13 **2195–2203**

ARTICLE

Dp260 disrupted mice revealed prolonged implicit time of the b-wave in ERG and loss of accumulation of β-dystroglycan in the outer plexiform layer of the retina

Shuhei Kameya[1,7], Eiichi Araki[2], Motoya Katsuki[3], Atsushi Mizota[4], Emiko Adachi[4], Kiyoshi Nakahara[5], Ikuya Nonaka[6], Shozo Sakuragi[7], Shin'ichi Takeda[1,*] and Yo-ichi Nabeshima[1,8]

[1]Department of Molecular Genetics and [6]Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187, Japan, [2]Department of Neurology, Neurological Institute, Faculty of Medicine, Kyushu University, Fukuoka, Japan, [3]Department of DNA Biology and Embryo Engineering, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [4]Department of Ophthalmology, Chiba University School of Medicine, Chiba, Japan, [5]Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto, Japan, [7]Department of Ophthalmology, Akita University School of Medicine, Akita, Japan and [8]Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan

Received May 27, 1997; Revised and Accepted September 11, 1997

Dp260 is a C-terminal isoform of dystrophin and is expressed specifically in the retina. Abnormal electroretinograms (ERG) in some Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) patients are likely linked to a disruption of Dp260. To clarify the importance of Dp260 in the retina, we examined dystrophin exon 52 knock-out mice, whose expression of Dp260 is impaired. We also confirmed the localization of Dp260 in the outer plexiform layer (OPL) of the retina. Disruption of Dp260 causes a change in the localization of β-dystroglycan, which is normally found in the OPL of the retina. This suggests a requirement for Dp260 for normal formation of the dystrophin–dystroglycan complex in the retina. Dp71, also expressed in the retina, was, however, not detected in the OPL. The difference in localization of Dp260 and Dp71 implies that the two isoforms have different functions. The dystrophin exon 52 knock-out mice had a prolonged implicit time of the b-wave in ERG, although no significant change was observed in amplitude. These ERG findings differed from those of DMD and BMD patients, especially with regard to amplitude of the b-wave, but make it clear that Dp260 is required for normal electrophysiology.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X chromosome-linked recessive disease, characterized by a progressive wasting of skeletal muscles (1). The gene associated with DMD consists of at least 79 exons spanning >2.4 Mb on the human X chromosome (2,3). The mRNA transcript of this gene in skeletal muscle measures 14 kb and encodes a 427 kDa rod-shaped, spectrin-like protein called dystrophin (4). Dystrophin is located on the cytoplasmic surface of the sarcolemma in normal skeletal muscle (5–7). However, its precise cellular functions and the mechanisms by which its absence leads to myodegenerative disorders are unknown.

There are several tissue-specific and developmentally regulated dystrophin isoforms generated through differential promotor usage and alternative splicing at the 3′-end of the gene. Brain-, muscle- and Purkinje cell-specific promoters are located in the 5′-end of the gene, each of which directs transcription through a different 5′ first exon (8,9). Four smaller isoforms, Dp71, Dp116, Dp140 and Dp260, are transcribed using internal promoters and also have unique first exons (10–13). Dp71 is found in many non-muscle tissues, including the brain. Dp116 and Dp140 are exclusively expressed in the peripheral and central nervous system respectively. Finally, Dp260 is a retina-specific isoform located in the outer plexiform layer (OPL) (13,14).

There have been recent reports of abnormal retinal transmission in boys with either Duchenne or Becker muscular dystrophy (15–19). Dark-adapted electroretinograms (ERG) for these boys recorded under scotopic testing conditions have revealed normal a-waves, but b-waves with reduced amplitude. Under dark-adapted

*To whom correspondence should be addressed. Tel/Fax: +81 423 46 3864; Email: takeda@ncnaxp.ncnp.go.jp

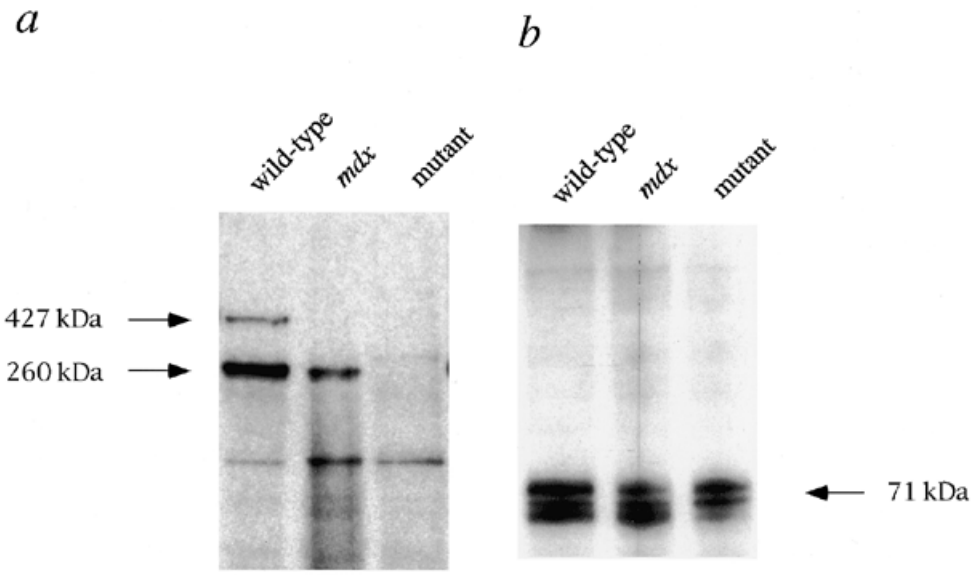

Figure 1. Identification of full-length dystrophin and its isoforms in the retina. Western blot analysis of retinal extract from wild-type, *mdx* and dystrophin exon 52 knock-out mice detected by anti-dystrophin monoclonal antibody MAB1694. (**a**) In a SDS–polyacrylamide gel (6%) the presence of full-length dystrophin and Dp260 was detected. Full-length dystrophin is present in wild-type mice, whereas Dp260 is present in wild-type and *mdx* mice. In mutant mice neither full-length dystrophin nor Dp260 was detected. Single bands of molecular weight 160 kDa, which appears in all three lanes, are non-specific. (**b**) In a SDS–polyacrylamide gel (8%) expression of Dp71 was detected. Dp71 is present in wild-type, *mdx* and mutant mice. Staining of the lowest band around 71 kDa was non-specific, since only the secondary antibody identified this band.

conditions ON bipolar cells depolarize, whereas OFF bipolar cells hyperpolarize in response to light. These reactions cause an increase in extracellular potassium concentration in the OPL (20). Subsequently, Müller cells, located in the OPL, take in potassium and transport it through the cell body, later to release it into the vitreous humour. This potassium flux corresponds to a positive polarity when recorded as a b-wave in ERG (21,22). The reduced b-wave in some DMD and BMD patients implies that full-length dystrophin or its isoforms is involved in the generation of b-waves in ERG under dark-adapted conditions.

The full-length dystrophin expressed in the retina is mainly of the brain type (23), but ERG from *mdx* mice show normal b-waves despite the absence of full-length dystrophin in these mice (16). DMD and BMD patients, who have deletions in the 5′-end of the DMD gene, reveal normal or nearly normal phenotypes in ERG, whereas patients who have deletions in exons 44–53 of the gene have severely abnormal phenotypes according to ERG (18). In the latter patients expression of Dp260 can be deficient, since the promoter of the gene that encodes this isoform exists between exons 29 and 30, making its disruption by the mutation likely. It appears that Dp260 is required for generation of normal b-waves, a hypothesis consistent with the finding that mdx^{Cv3} mice (24), having neither Dp260 nor Dp71, demonstrate attenuated amplitude and prolonged implicit time of b-waves.

Recently Araki *et al*. generated dystrophin exon 52 knock-out mice using the gene targeting technique (25). Disruption of exon 52 of the DMD gene caused the absence of full-length dystrophin in skeletal muscle, Dp260 in the retina and Dp140 in the brain respectively. However, expression of Dp116 and Dp71 was maintained in the sciatic nerve. The mouse revealed not only muscle degeneration and regeneration, but also hypertrophy of limb muscle, which was not observed in *mdx* mice.

To determine the importance of Dp260 for normal b-wave production, we investigated the isoforms of dystrophin expressed in the OPL of dystrophin exon 52 knock-out mice. This knock-out mouse revealed prolonged implicit time and normal amplitude of the b-wave in ERG, although immunoreactivity of full-length dystrophin and Dp260 in the OPL are completely missing.

In this knock-out mouse we also investigated expression of metabotropic glutamate receptor subtype 6 (mGluR6) in the OPL. mGluR6 is restrictedly expressed in ON bipolar cells (26) and is essential for the ON response in visual synaptic transmission (27). mGluR6 becomes concentrated at the post-synaptic site of bipolar cell dendrites during development and is reversed when photoreceptors degenerate in a mutant rat with retinal dystrophy. Null mutant mice of mGluR6 demonstrate no b-wave in ERG. Dystrophin and one of its isoforms might anchor the mGluR6 molecule in ON bipolar cells. The expression pattern of mGluR6 is not altered in the knock-out mouse.

RESULTS

Identification of dystrophin isoforms in the retina

Dystrophin exon 52 knock-out mice were recently generated by Araki *et al*. (25). We examined retinal extract from wild-type, *mdx* and dystrophin exon 52 knock-out mice using the mouse monoclonal antibody MAB1694, specific for the last 17 amino acids of the C-terminal region of dystrophin (Fig. 1). In wild-type mice the antibody identified three distinct isoforms having estimated molecular weights of 427, 260 and 71 kDa. A single band at 427, a major band at 260 and two bands around 71 kDa corresponded to full-length dystrophin, Dp260 and Dp71 respectively. The doublet bands, identified as Dp71, resulted from

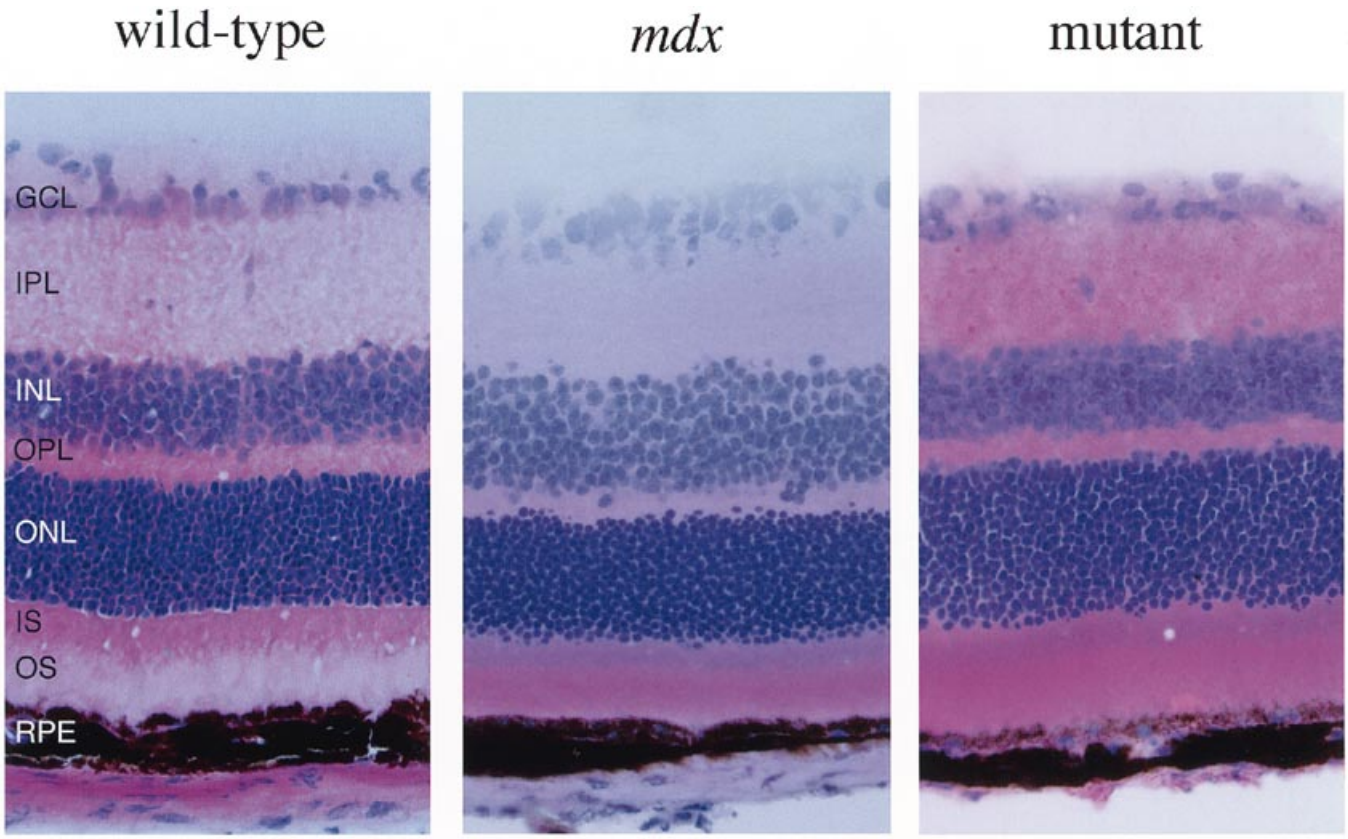

Figure 2. Histological analyses of the retinas of wild-type, *mdx* and dystrophin exon 52 knock-out mice. Vertical sections of the retina from wild-type, *mdx* and mutant mice were stained with hematoxylin and eosin. Each layer is designated as follows: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment of photoreceptors; OS, outer segment of photoreceptors; RPE, retinal pigment epithelium. Well-organized and layered structures of the retina are maintained in *mdx* and mutant mice.

alternative splicing around exons 71–78. We could not detect Dp140 and Dp116 in the retina of wild-type mice, though both isoforms were present in Western blots using a rabbit polyclonal antibody, which recognizes the last 17 amino acids of dystrophin (28). We also examined retinal extract using the antibody MANDRA1, raised against the C-terminal domain of dystrophin. Again, this antibody could detect neither Dp140 nor Dp116 in the retina of wild-type mice (data not shown). In the retina of *mdx* mice Dp260 and Dp71 were present, while full-length dystrophin was undetectable, in agreement with a previous report (13). Dp71 was also preserved in the retina of the dystrophin exon 52 knock-out mice, but both full-length dystrophin and Dp260 were absent in this case; these mice proved to be full-length dystrophin- and Dp260-deficient mutants.

Localization of dystrophin isoforms in the mutant retina

Hematoxylin and eosin staining revealed the retina of the mutant mice to be normally organized in an orderly layered anatomical arrangement, similar to wild-type and *mdx* mice (Fig. 2). To determine the localization of dystrophin and its isoforms in the mutant, a series of retinal sections from wild-type, *mdx* and mutant mice was immunostained with a battery of antibodies against different regions of dystrophin. Using the monoclonal antibody MAB1692, which recognizes the middle part of the rod domain and thus identifies solely full-length dystrophin, we have achieved restricted and punctate staining in the OPL of wild-type mice, while the same staining was undetectable in the OPL of *mdx* and mutant mice (Fig. 3a–c). These results indicate that full-length dystrophin is confined to the OPL. We then carried out immunostaining with the polyclonal antibody P-23a, raised against a part of the second half of the rod domain, which recognizes full-length dystrophin and Dp260. Staining in the OPL is present in wild-type and *mdx* mice, but not in the mutant mice (Fig. 3d–f). This staining indicates that Dp260 is also confined to the OPL. Using the monoclonal antibody MAB1694, raised against the C-terminus last 17 amino acids of dystrophin, which recognizes all three dystrophin isoforms, we found staining in the OPL of wild-type and *mdx* mice, but not in the mutant mice (Fig. 3g–i). Western blot analysis using the same antibody suggested that Dp71 is expressed in the retina of the mutant; however, there was no concentrated staining in the mutant OPL. Retinal blood vessels of wild-type, *mdx* and mutant mice were equally stained and this finding suggested expression of Dp71 around the blood vessels. We also immunostained sections with the monoclonal antibody MANDRA1, raised against the C-terminal domain of dystrophin, which recognizes the three dystrophin isoforms; the same pattern obtained with MAB1694 was found in the OPL of wild-type, *mdx* and mutant mice (Fig. 3j–l). Expression of Dp140 was also disrupted in the mutant (25) and thus it is intriguing to know whether Dp140 is expressed in the mouse retina or not. In our Western blots, however, there was no trace of Dp140

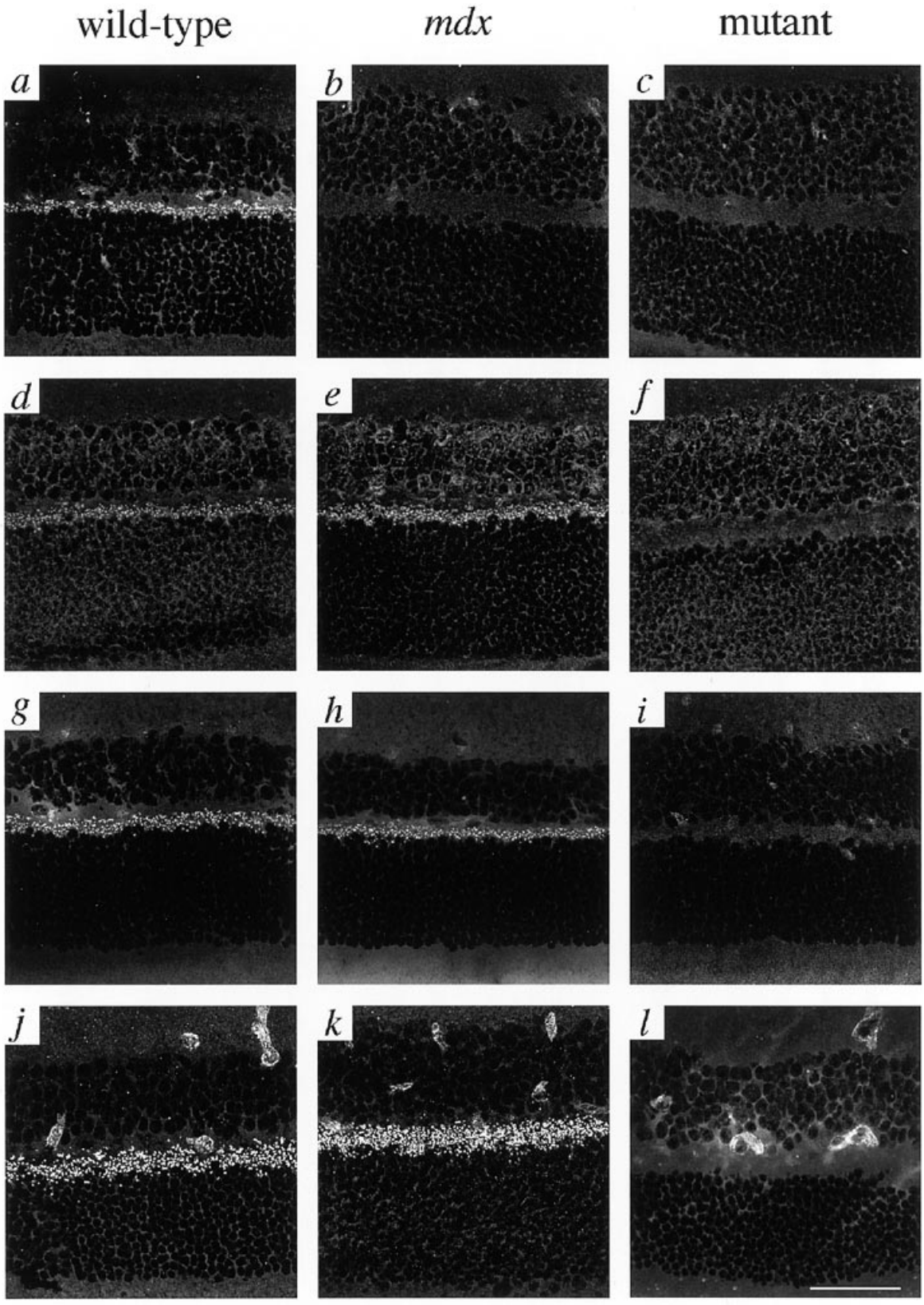

Figure 3. Immunohistochemical analyses of retinas from wild-type, *mdx* and dystrophin exon 52 knock-out mice using several antibodies against dystrophin. Sections from wild-type (**a**, **d**, **g** and **j**), *mdx* (**b**, **e**, **h** and **k**) and mutant mice (**c**, **f**, **i** and **l**) were immunostained with the following antibodies: MAB1692 (a–c); P-23a (d–f); MAB1694 (g–i); MANDRA1 (j–l). See Results. Scale bar 50 μm.

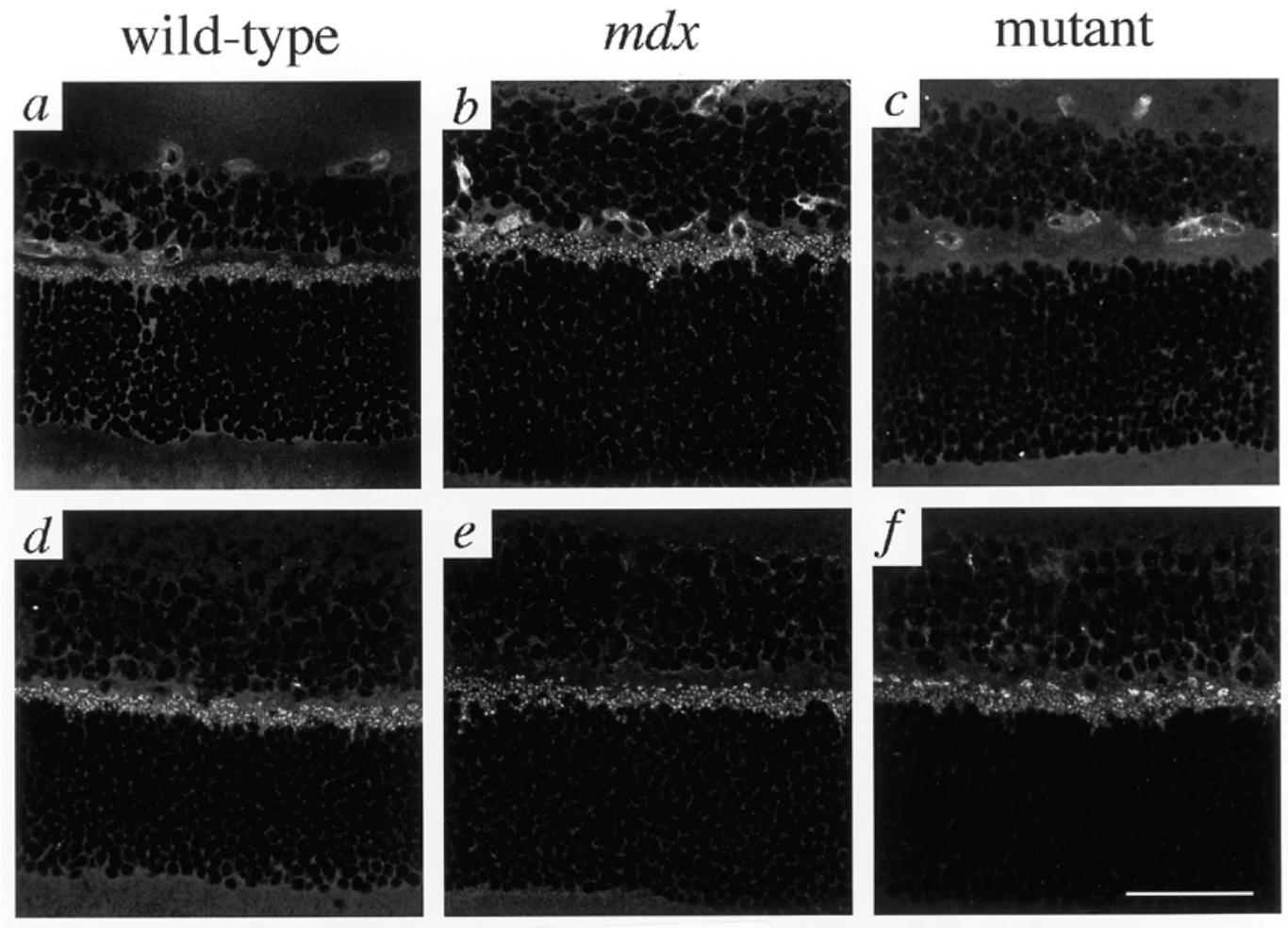

Figure 4. Immunohistochemical analyses of retinas from wild-type, *mdx* and dystrophin exon 52 knock-out mice using antibodies against β-dystroglycan and mGluR6. Sections from wild-type (**a** and **d**), *mdx* (**b** and **e**) and mutant mice (**c** and **f**) were immunostained with antibody against β-dystroglycan (a–c) and mGluR6 (d–f). Punctate immunoreactivity of β-dystroglycan is seen in the OPL of wild-type and *mdx* mice, but is absent in the mutant mice. No gross changes in the immunostaining of mGluR6 were observed in *mdx* and mutant mice. Scale bar 50 μm.

or Dp116 in retinal extracts (Fig. 1) and expression of both isoforms has not been suggested in our immunohistochemical study.

Localization of β-dystroglycan and mGluR6 in the mutant retina

To determine whether there is β-dystroglycan expression in the mutant retina, we examined retinal sections with an antibody against β-dystroglycan. Restricted and punctate staining of β-dystroglycan was detected in the OPL of wild-type and *mdx* mice, but was absent in the mutant OPL (Fig. 4a–c). This result revealed that full-length dystrophin or Dp260 is necessary for localization of β-dystroglycan in the OPL and suggested that without full-length dystrophin or Dp260, dystrophin-associated protein cannot accumulate in the OPL. This antibody stained retinal blood vessels of wild-type, *mdx* and mutant mice, as did MAB1694 and MANDRA1.

To determine whether accumulation of mGluR6 is altered in the absence of Dp260, thus causing absence of the dystrophin–dystroglycan complex, we investigated expression of mGluR6 in the OPL (Fig. 4d–f). Punctate immunoreactivity was observed in the OPL of wild-type, *mdx* and mutant mice. The pattern and amount of mGluR6 detected in the OPL were indistinguishable among these mice.

Table 1. Electroretinographic findings in wild-type and mutant mice

	Wild-type	Mutant	*P*-value
Amplitude (μV)			
a-wave	300 ± 54.0	425 ± 58.6	0.013
b-wave	988 ± 153.3	955 ± 92.5	0.704
b/a ratio	3.36 ± 0.706	2.26 ± 0.201	0.049
Implicit time (ms)			
a-wave	23.4 ± 3.89	30.1 ± 2.23	0.014
b-wave	46.7 ± 2.35	61.8 ± 0.201	0.0001

The values are mean ± SD at stimulus intensity level of –2.5 log NDF.

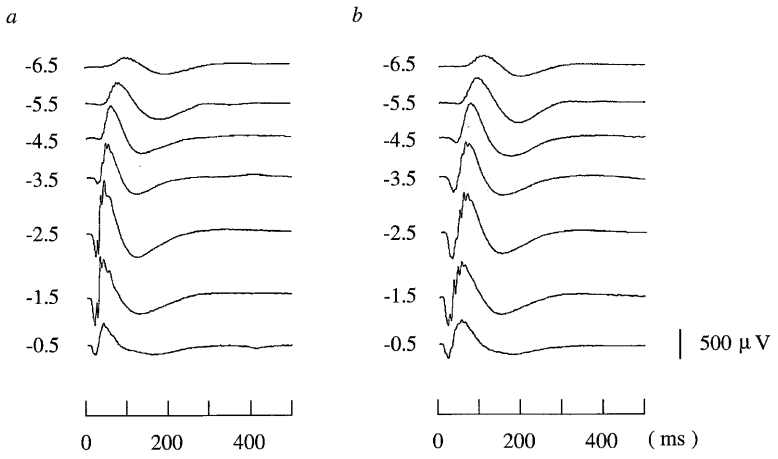

Figure 5. Representative ERGs of wild-type and dystrophin exon 52 knock-out mice. ERGs are in response to different stimulus intensity levels and were recorded from wild-type (**a**) and mutant mice (**b**) in dark-adapted conditions. The numbers represent the value of the NDFs used to reduce the full-intensity stimulus. In the mutant mouse the implicit time of the b-wave is prolonged.

ERG findings

To determine the influence of Dp260 depletion on visual synaptic transmission at the OPL, we compared the ERGs of wild-type mice with those of mutant mice (Fig. 5). The b-wave amplitude of the ERG of mutant mice did not differ from that of wild-type mice (Fig. 6a). However, the implicit time of the b-wave of mutant mice was significantly increased across the entire range of stimulus intensity levels (Fig. 6b). At a stimulus intensity level of –2.5 logNDF the amplitude and the implicit time of the a-wave were greater and longer in mutant mice than in wild-type mice (Table 1). Consequently, the b/a amplitude ratio was smaller in mutant mice compared with wild-type mice. (Table 1). We note that an increased amplitude and prolonged implicit time were not observed after relatively strong stimuli (Fig. 6c and d). The ERGs of *mdx* mice, which are of B10 background, showed no difference in amplitude and implicit time of the a- and b-waves compared with control B10 mice.

DISCUSSION

In this study we confirmed localization of Dp260 in the OPL of the retina. Although previous immunohistochemical analysis of *mdx* mice suggested localization of Dp260 in the OPL (13), we obtained further evidence by comparing *mdx* mice with dystrophin exon 52 knock-out mice in another immunohistochemical analysis. The pattern in mutants having neither full-length dystrophin nor Dp260 indicates that the punctate pattern in the OPL of *mdx* mice was due to expression of Dp260.

Dystrophin exon 52 knock-out mice did not express β-dystroglycan in the OPL, unlike wild-type and *mdx* mice. α- and β-dystroglycan span the sarcolemma of skeletal muscle to provide a link between the subsarcolemmal cytoskeleton and the extracellular matrix; they form a large oligomeric complex with dystrophin (29,30). In the skeletal muscle of *mdx* mice and DMD patients the absence of dystrophin leads to a reduction in expression of α- and β-dystroglycan. Dystrophin has been shown to form a complex in the retina. Co-localization of dystrophin and β-dystroglycan has been discovered in the mouse and human OPL (28,31). The absence of β-dystroglycan in the OPL of the mutant mice strongly suggests disruption of a dystrophin–dystroglycan complex and also implies that Dp260 allows localization of β-dystroglycan at the OPL, although there is no full-length dystrophin in the region.

Compared with wild-type mice the ERGs of dystrophin exon 52 knock-out mice display increased implicit time of the b-wave without any difference in amplitude, larger amplitude and increased implicit time of the a-wave and a lower b/a amplitude ratio. A greater amplitude and increased implicit time of the a-wave might be explained as a secondary effect of delayed generation of the b-wave. This is feasible because no abnormality of the a-wave resulted after high intensity stimuli, which, nonetheless, cause a smaller b-wave. There also remains the possibility that the change in the a-wave stems from functional defects at the photoreceptor level. The delay in the b-wave implicit time indicates a molecular anomaly involving the release of L-glutamate from photoreceptors and the release of potassium ions from ON bipolar cells, all of which take place at the OPL (20).

Compared with *mdx*^{Cv3} mice, which reveal delayed implicit time and reduced amplitude in the b-wave (24), dystrophin exon 52 knock-out mice show normal amplitude of the b-wave. The difference between the *mdx*^{Cv3} mouse and this mutant mouse suggests that Dp71 plays an important role in generation of the b-wave in ERG. From our observations on Western blots and immunohistochemistry using a battery of dystrophin antibodies it is very difficult to determine the contribution of Dp140 and Dp116 in generation of the ERG b-wave. On the other hand, Dp71 was identified in the retina of the mutant mice using Western blot analysis. Expression, however, was not detected in the OPL by immunohistochemical analysis. The absence of β-dystroglycan in the OPL strengthened the idea that Dp71 is not concentrated in large amounts in the mutant OPL. Jung *et al*. (32) examined the subcellular localization of Dp71 in the rat brain and found that it appeared mainly in synaptic plasma membranes and microsomes and not in the mitochondrial fraction. Dp71 has also been detected in cultured glial cells (33). Retinal glial cells which might express Dp71 include Müller cells and astrocytes. Positive dystrophin staining around retinal blood vessels might reflect expression of Dp71 in Müller cells and astrocytes (34). Interestingly, Müller

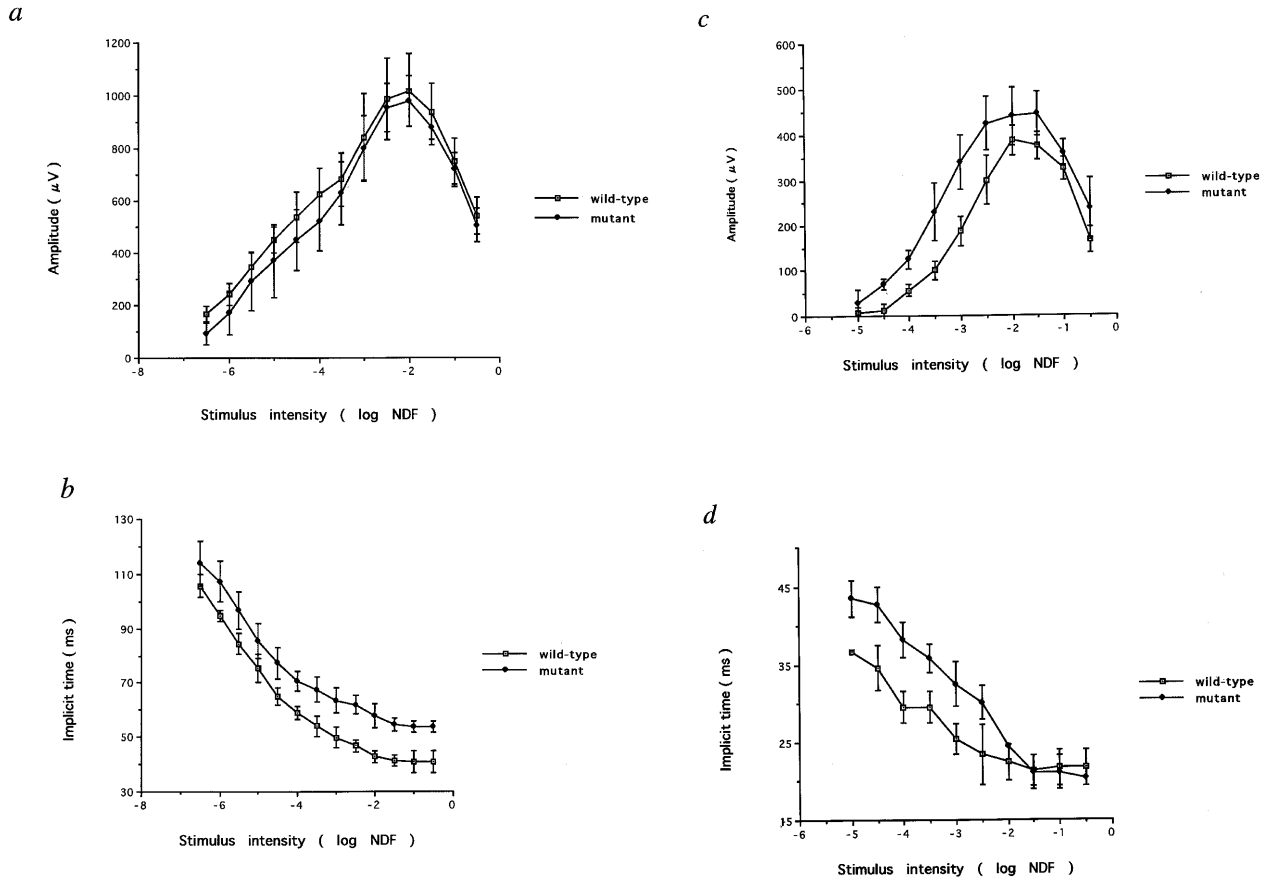

Figure 6. The amplitude and the implicit time of the a- and b-waves. The amplitude (**a**) and the implicit time (**b**) of the b-wave of wild-type and dystrophin exon 52 knock-out mice at –6.5 to –0.5 logNDF stimulus intensity levels (mean ± SD). No significant difference is observed between the amplitude of wild-type and mutant mice at all stimulus intensity levels. Mutant mice have prolonged implicit time compared with wild-type mice at all stimulus intensity levels. The amplitude (**c**) and the implicit time (**d**) of the a-wave at –6.5 to –0.5 logNDF stimulus intensity levels (mean ± SD) are also shown.

cells have been suggested to be involved in generation of the b-wave in ERG (21,22).

Since DMD patients display markedly reduced amplitude of the b-wave, it is difficult to measure the precise implicit time of the b-wave. Dystrophin isoforms can be detected in the retina of DMD/BMD patients with abnormal ERG; however, it is assumed that expression of Dp260 is subject to loss, as evidenced by the deletion pattern of the DMD gene (18). The reason why similar genetic defects have different phenotypes in DMD/BMD patients and the dystrophin exon 52 knock-out mice of this study is unknown, but species differences between the mouse and human are likely to cause the divergent phenotypic expression of both. In the *mdx* mouse regenerated fibers, except those of the diaphragm, are resistant to further degradation. Regenerated fibers are, however susceptible to degradation in DMD patients (35).

It is possible that mGluR6 is also involved in generation of the ERG b-wave, since disruption of the gene causes disappearance of the b-wave (27). This study clearly indicates that accumulation of mGluR6 is independent of expression of full-length dystrophin/Dp260 in the OPL and of formation of the dystrophin–dystroglycan complex. In the neuromuscular junction utrophin, the autosomal homolog of dystrophin, is closely associated with the nicotinic acetylcholine receptor (nAChR) throughout development and in the adult, but it is not essential for nAChR clustering (36,37). Instead, other post-synaptic molecules, like rapsin (38), agrin (39) and MuSK (40), are important for this function. Similarly, accumulation of mGluR6 in the OPL may also be regulated by molecules other than dystrophin, but they have not yet been identified. Dystrophin exon 52 knock-out mice revealed b-wave abnormalities without an alteration in mGluR6, implying that full-length dystrophin/Dp260 affect generation of the b-wave independently of accumulation or maintenance of mGluR6.

Formation of the dystrophin–glycoprotein complex has been extensively investigated in skeletal muscle. In this report we have shown that accumulation of β-dystroglycan depends on expression of full-length dystrophin and its isoforms, in particular Dp260, in the OPL through use of dystrophin exon 52 knock-out mice. The molecular background of the generation of ERG and especially the relationship between dystrophin and its isoforms and ERG can be clarified by further study.

MATERIALS AND METHODS

Animals

The generation of dystrophin exon 52 knock-out mice was described in detail by Araki *et al*. (25). In brief, to construct the

targeting vector a 2.5 kb *Hinc*II fragment containing exon 52 of the DMD gene was replaced with the neomycin resistance gene. The targeting vector was electroporated into CCE ES cells. Properly recombinated ES cells were cloned by positive–negative selection. Chimeric mice were obtained by injecting this clone into blastocysts of the C57BL/6J inbred strain. Heterozygous female F1 mice (X^{mdx52}/X) were crossed with C57BL/6J males to obtain dystrophin-deficient male mice. Littermate male mice of hemizygous mutant mice (X^{mdx52}/Y) were used as wild-type controls. ERGs for five mutants and four wild-type mice were performed. The average age and weight of the mice at the time of testing did not differ significantly among the two groups. The animals were kept on a 12 h light/dark cycle, then anesthetized by i.m. injection of a mixture of ketamine (11 mg/kg), xyladine (14 mg/kg) and urethane (500 mg/kg). All experiments conformed to the standards of the Association for Research in Vision and Ophthalmology Resolution on the use of animals in research.

Western blot analysis

To obtain samples of the mouse retina, the front hemisphere of the eyeball was removed so that the retina could be gently scraped off from the rear hemisphere with the help of a stereoscopic microscope. Protein fractions were then extracted using a reducing sample buffer (10% SDS, 70 mM Tris–HCl, 5% β-mercaptoethanol, 10 mM EDTA). Aliquots of 20 μg/lane retinal extract were separated on 6 or 8% SDS–polyacrylamide gels. The proteins were subsequently transferred to a PVDF membrane (Millipore) employing an ampage of 242 mA for 2 h. The blot was later incubated with the anti-dystrophin monoclonal antibody MAB1694 (Chemicon), which recognizes the final 17 amino acids of dystrophin. The antibody was used at a 1/200 dilution and the signal was detected using the enhanced chemiluminescence (Amersham) method and a 1/5000 dilution of the anti-mouse secondary antibody (Amersham).

Histology and immunohistochemistry

The eyes of mice were embedded in O.C.T. compound (Miles Inc.) and frozen in liquid nitrogen before 6 μm sections were cut and placed on poly(L-lysine)-coated slides. The slides were brought to room temperature, air dried for 1 h and acetone fixed for 10 min. For histological analysis the fixed sections were stained with hematoxylin and eosin. For immunohistochemistry the sections were blocked with 5% goat serum, 2% bovine serum albumin in 0.01 M phosphate-buffered saline at 37°C for 15 min, then incubated overnight at 4°C with the following primary antibodies, diluted in 0.01 M phosphate-buffered saline containing 5% goat serum and 2% bovine serum albumin: P-23a, a rabbit polyclonal antibody raised against the rod domain of dystrophin (amino acids 2360–2409), provided by Dr Yoshida (National Institute of Neuroscience, Tokyo) and used at a 1/50 dilution; MAB1694 (Chemicon), a hybridoma supernatant containing a monoclonal antibody to the last 17 amino acids of the dystrophin C-terminus, used at a 1/500 dilution; MAB1692 (Chemicon), a hybridoma supernatant containing a monoclonal antibody against the rod part of human dystrophin (amino acids 1181–1388), used at a 1/2 dilution; MANDRA1 (Sigma), ascites fluid containing a monoclonal antibody against amino acids 3558–3684 of human dystrophin, used at a 1/100 dilution; anti-β-dystroglycan (Novocastra), a hybrydoma supernatant containing a monoclonal antibody, used at a 1/10 dilution; mGluR6 antibody, a rabbit polyclonal antibody against the C-terminal sequence of rat mGluR6, used at a 1/300 dilution. Following incubation with primary antibodies, the sections were washed three times for 30 min in phosphate-buffered saline containing 0.5% skim milk. The secondary antibodies used were as follows: for P-23a and mGluR6, FITC-conjugated goat antibody against rabbit (Tago Immunologicals); for monoclonal antibody MAB1692, MAB1694, MANDRA1 and β-dystroglycan, FITC-conjugated rabbit antibody against mouse (Tago Immunologicals).

ERG

A cotton wick electrode placed on the cornea was partnered with another electrode attached s.c. to the nasal bone. The stimulus light was from a 150 W quartz-halogen lamp. The light was collected and focused onto a 3 mm diameter fiber optic bundle. The edge of the bundle was placed 0.5 cm from the corneal plane. Illuminance of the unattenuated stimulus on the surface of the eye was 140 000 lux and neutral density filters (NDF) were used to reduce the stimulus intensity. The 20 ms duration of the stimulus was controlled by an electromagnetic shutter. After 30 min dark adaptation, responses were recorded beginning with the lowest stimulus intensity. The responses were amplified by a pre-amplifier that had a band width between 1.5 and 100 Hz. Single flash ERGs were recorded. The a-wave amplitude was determined from the baseline to the bottom of the a-wave. The b-wave amplitude was determined from the bottom of the a-wave to the top of the b-wave. The implicit time of the a-wave and the b-wave was defined as the duration between the rise of the stimulus and the peak of the wave.

ACKNOWLEDGEMENTS

We thank Dr M.Yoshida for supplying the antibody P-23a. This work was partly supported by grants for Nervous and Mental Disorders from the Ministry of Health and Welfare. The authors thank Mr Daniel North for his review of this manuscript.

ABBREVIATIONS

BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; ERG, electroretinogramm; GluR6, metabotropic glutamate receptor subtype 6; nAChR, nicotinic acetylcholine receptor; NDF, neutral density filter; OPL, outer plexiform layer.

REFERENCES

1. Moser,H. (1984) Duchenne muscular dystrophy: pathogenic aspects and genetic prevention. *Hum. Genet*., **66**, 17–40.
2. Koenig,M., Hoffman,E.P., Bertelson,C.J., Monaco,A.P., Feener,C. and Kunkel,L.M. (1987) Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell*, **50**, 509–517.
3. Roberts,R.G., Coffey,A.J., Bobrow,M. and Bentley,D.R. (1992) Determination of the exon structure of the distal portion of the dystrophin gene by vectorette PCR. *Genomics*, **13**, 942–950.
4. Koenig,M., Monaco,A.P. and Kunkel,L.M. (1988) The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell*, **53**, 219–226.
5. Arahata,K., Ishiura,S., Ishiguro,T., Tsukahara,T., Suhara,Y., Eguchi,C., Ishihara,T., Nonaka,I., Ozawa,E. and Sugita,H. (1988) Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy peptide. *Nature*, **333**, 861–863.

6. Zubrzycka-Gaarn,E.E., Bulman,D.E., Karpati,G., Burghes,A.H.M., Belfall,B., Klamut,H.J., Talbot,J., Hodges,R.S., Ray,P.N. and Worton,R.G. (1988) The Duchenne muscular gene product is localized in the sarcolemma of human skeletal muscle. *Nature*, **333**, 466–469.
7. Watkins,S.C., Hoffman,E.P., Slayter,H.S. and Kunkel,L.M. (1988) Immunoelectron microscopic localization of dystrophin in myofibers. *Nature*, **333**, 863–866.
8. Chelly,J., Hamard,G., Koulakoff,A., Kaplan,J.C., Kahn,A. and Berwald Netter,Y. (1990) Dystrophin gene transcribed from different promoters in neuronal and glial cells. *Nature*, **344**, 64–65.
9. Gorecki,D., Monaco,A., Derry,J., Walker,A., Barnard,E. and Barnard,P. (1992) Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters. *Hum. Mol. Genet*., **1**, 505–510.
10. Lederfein,D., Levy,Z., Augier,N., Mornet,D., Morris,G., Fuchs,O. and Yaffe,D. (1992) A 71-kilodalton protein is a major product of the Duchenne muscular dystrophy gene in brain and other nonmuscle tissues. *Proc. Natl. Acad. Sci. USA*, **89**, 5346–5350.
11. Byers,T., Lidov,H. and Kunkel,L. (1993) An alternative dystrophin transcript specific to peripheral nerve. *Nature Genet*., **4**, 77–81
12. Lidov,H.G.W., Selig,S. and Kunkel,L.M. (1995) Dp140: a novel 140 kDa CNS transcript from the dystrophin locus. *Hum. Mol. Genet*., **4**, 329–335.
13. D'Souza,V.N., Nguyen,T.M., Morris,G.E., Karges,W., Pillers,D.A. and Ray,P.N. (1995) A novel dystrophin isoform is required for normal retinal electrophysiology. *Hum. Mol. Genet*., **4**, 837–842.
14. Miike,T., Miyatake,M., Zhao,J., Yoshioka,K. and Uchino,M. (1989) Immunohistochemical dystrophin reaction in synaptic regions. *Brain Dev.*, **11**, 344–346.
15. Pillers,D.M., Bulman,D.E., Weleber,R.G., Sigesmund,D.A., Musarella,M.A., Powell,B.R., Murphey,W.H., Westall,C., Panton,C., Becker,L.E., Worton,R.G. and Ray,P.N. (1993) Dystrophin expressed in the retina is required for normal function as defined by electroretinography. *Nature Genet*., **4**, 82–86.
16. Cibis,G.W., Fitzgerald,K.M., Harris,D.J., Rothberg,P.G. and Rupani,M. (1993) The effect of dystrophin gene mutations on the ERG in mice and humans. *Invest. Ophthalmol. Vision Sci*., **34**, 3646–3652.
17. Fitzgerald,K.M., Cibis,G.W., Giambrone,S.A. and Harris,D.J. (1994) Retinal signal transmission in Duchenne muscular dystrophy: evidence for dysfunction in the photoreceptor/depolarizing bipolar cell pathway. *J. Clin. Invest*., **93**, 2425–2430.
18. Sigesmund,D.A., Weleber,R.G., Pillers,D.M., Westall,C.A., Panton,C.M., Powell,B.R., Heon,E., Murphey,W.H., Musarella,M.A. and Ray,P.N. (1994) Characterization of the ocular phenotype of Duchenne and Becker muscular dystrophy. *Ophthalmology*, **101**, 856–865.
19. DeBecker,I., Riddell,D.C., Dooley,J.M. and Tremblay,F. (1994) Correlation between electroretinogram findings and molecular analysis in the Duchenne muscular dystrophy phenotype. *Br. J. Ophthalmol*., **78**, 719–722.
20. Stockton,R.A. and Slaughter,M.M. (1989) The b-wave of the electroretinogram: a reflection of ON bipolar cell activity. *J. Gen. Physiol*., **61**, 1233–1243.
21. Newman,E.A. and Odette,L.L. (1984) Model of electroretinogram b-wave generation: a test of the K^+ hypothesis. *J. Neurophysiol*., **51**, 164–182.
22. Wen,R. and Oakley,B. (1990) K^+-evoked Müller cell depolarization generates b-wave of electroretinogram in toad retina. *Proc. Natl. Acad. Sci. USA*, **87**, 2117–21.
23. Tamura,T., Yoshioka,K., Jinno,Y., Niikawa,N. and Miike,T. (1993) Dystrophin isoforms expressed in the mouse retina. *J. Neurol. Sci*., **115**, 214–218.
24. Pillers,D.M., Weleber,R.G., Woodward,W.R., Green,D.G., Chapman,V.M. and Ray,P.N. (1995) *mdx*^{Cv3} mouse is a model for electroretinography of Duchenne/Becker muscular dystrophy. *Invest. Ophthalmol. Vision Sci*., **36**, 462–466.
25. Araki,E., Nakamura,K., Nakao,K., Kameya,S., Kobayashi,O., Nonaka,I., Kobayashi,T. and Katsuki,M. (1997) Targeted disruption of exon 52 in the mouse dystrophin gene induced muscle degeneration similar to that observed in Duchenne muscular dystrophy. *Biochem. Biophys. Res. Commun*., in press.
26. Nomura,A., Shigemoto,R., Nakamura,Y., Okamoto,N., Mizuno,N. and Nakanishi,S. (1994) Developmentally regulated postsynaptic localization of metabotropic glutamate receptor in rat rod bipolar cells. *Cell*, **77**, 361–369.
27. Masu,M., Iwakabe,H., Tagawa,Y., Miyoshi,T., Yamashita,M., Fukuda,Y., Sasaki,H., Hiroi,K., Nakamura,Y., Shigemoto,R., Takada,M., Nakamura,K., Nakao,K., Katsuki,M. and Nakanishi,S. (1995) Specific deficit of the ON response in visual transmission by targeted disruption of the mGluR6 gene. *Cell*, **80**, 757–765.
28. Montanaro,S., Carbonetto,S., Campbell,K.P. and Lindenbaum,M. (1995) Dystroglycan expression in the wild-type and *mdx* mouse neural retina: synaptic co-localization with dystrophin, dystrophin-related protein but not laminin. *J. Neurosci. Res*., **42**, 528–538.
29. Ervasti,J.M. and Campbell,K.P. (1991) Membrane organization of the dystrophin–glycoprotein complex. *Cell*, **66**, 1121–1131.
30. Campbell,K.P. (1995) Three muscular dystrophies: loss of cytoskeleton–extracellular matrix linkage. *Cell*, **80**, 675–679.
31. Drenckhahn,D., Holbach,M., Ness,W., Schmitz,F. and Anderson,L.V.B. (1996) Dystrophin and the dystrophin-associated glycoprotein, β-dystroglycan, co-localize in photoreceptor synaptic complexes of the human retina. *Neuroscience*, **72**, 605–612.
32. Jung,D., Filliol,D., Metz-Boutigue,M.H. and Rendon,A. (1993) Characterization and subcellular localization of the dystrophin-protein 71 (Dp71) from brain. *Neuromusc. Disorders*, **3**, 515–518.
33. Schofield,J.N., Blake,D.J., Simmons,C., Morris,G.E., Tinsley,J.M., Davies,K.E. and Edwards,Y.H. (1994) Apo-dystrophin-1 and apo-dystrophin-2, products of the Duchenne muscular dystrophy locus: expression during mouse embryogenesis and in cultured cell line. *Hum. Mol. Genet*., **3**, 1309–1316.
34. Horstmär,H., Makarov,F., Dreher,Z., Driel,D.V., Chan-Ling,T. and Stone,J. (1991) Structure of the macroglia of the retina: sharing and division of labour between astrocytes and Müller cells. *J. Comp. Neurol*., **313**, 587–603
35. Cullen,M.J. and Jaros,E. (1988) Ultrastructure of the skeletal muscle in the X chromosome-linked dystrophic (*mdx*) mouse. Comparison with Duchenne muscylar dystrophy. *Acta Neuropathol*., **77**, 69–81.
36. Grady,R.M., Merlie,J.P. and Sanes,J.R. (1997) Subtle neuromuscular defects in utrophin-deficient mice. *J. Cell Biol*., **136**, 871–882.
37. Deconinck,A.E., Potter,A.C., Tinsley,J.M., Wood,S.J., Vater,L., Young,C., Metzinger,L., Vincent,A., Slater,C.R. and Davies,K.E. (1997) Postsynaptic abnormalities at the neuromuscular junctions of utrophin-deficient mice. *J. Cell Biol*., **136**, 883–894.
38. Gautam,M., Noakes,P.G., Mudd.J., Nichol,M., Chu,G.C., Sanes,J.R. and Merlie,J.P. (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsin-deficient mice. *Nature*, **377**, 232–236.
39. Gautam,M., Noakes,P.G., Moscoso,L.,Rupp,F., Scheller,R.H., Merlie,J.P. and Sanes,J.R. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* **85**, 525–535.
40. DiChiara,T.M., Bowen,D.C., Valenzuela,D.M., Simmons,M.V., Poueymirou,W.T., Thomas,S., Kinetz,E., Compton,D.L., Rojas,E., Park,J.S., Smith,C., DiStefano,P.S., GlassD.J., Burden,S.J. and Yancopoulos,G.D. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation *in vivo*. *Cell*, **85**, 501–512.