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Dystroglycan Expression in the Wild Type and *mdx* Mouse Neural Retina: Synaptic Colocalization With Dystrophin, Dystrophin-Related Protein But Not Laminin

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Alpha- and β-dystroglycan (α- and β-DG) are members of a dystrophin-associated glycoprotein complex (DGC) in skeletal muscle which binds to agrin and laminin, and has been postulated to be involved in myoneural synapse formation. The absence of functional dystrophin in Duchenne muscular dystrophy (DMD) and in one of its animal models, the *mdx* mouse, leads to a reduction of α- and β-DG in muscle, and is often associated with mental retardation and abnormal retinal synaptic transmission in DMD. Using immunohistochemistry, we find that α- and β-DG are expressed in the outer plexiform layer of both wild type and mdx retina, where both dystrophin and dystrophin-related protein (DRP), but not laminin are present. In situ hybridization identifies two neuronal populations, photoreceptors and retinal ganglion cells, that express DG mRNA. α- and β-DG are also expressed in the inner limiting membrane and around blood vessels where they colocalize with laminin and DRP. Western blot analysis revealed the expression of several dystrophin isoforms in wild type and mdx retina, possibly explaining the unaltered expression of α- and β-dystroglycan in the mdx central nervous system (CNS). Our results support the hypothesis that α- and β-DG can interact with dystrophin and DRP in the CNS and perform functions analogous to those of the DGC in muscle.

Key words: dystroglycan, *mdx* mouse, retina, synapse

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe, X-linked myodegenerative disorder whose primary defect results from mutations in the gene for dystrophin. While dystrophin is relatively abundant in muscle where it forms part of the submembranous cytoskeleton, its precise cellular functions and the mechanisms by which its absence leads to myodegenerative disorders remain obscure. Recent studies have characterized an oligomeric transmembrane complex in muscle that binds dystrophin and dystrophin-related protein (DRP; utrophin) intracellularly, and interacts with several extracellular matrix (ECM) proteins such as laminin, merosin and agrin (Douville et al., 1988; Ibraghimov-Beskrovnaya et al., 1992; Matsumura et al., 1992; Ervasti and Campbell, 1993; Gee et al., 1993, 1994; Campanelli et al. 1994; Bowe et al., 1994; Yamada et al., 1994). This dystrophin-associated glycoprotein complex (DGC) comprises five transmembrane proteins of 35 (35 DAG), 43 (β-dystroglycan and A3b), 50 (adhalin), and 25 kDa (25 DAP), one extracellular membrane-associated glycoprotein of 156 kDa (α-dystroglycan), and one intracellular membrane-associated protein of the syntrophin family (59 kDa; Ibraghimov-Beskrovnaya et al., 1992; Adams et al., 1993; Ahn et al., 1994). β-dystroglycan (β-DG) and β1-syntrophin have both been shown to interact directly with different regions of the carboxyl-terminal of dystrophin (Suzuki et al., 1994), while α-dystroglycan (α-DG) is responsible for high affinity binding to the ECM molecules laminin and agrin. α- and β-DG are derived from a common precursor protein which is cleaved during or shortly after translation, and are therefore found to be coexpressed in a variety of tissues (Ibraghimov-Beskrovnaya et al., 1992).

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The absence of dystrophin in DMD and in one of its animal models, the mdx mouse, leads to a reduction of expression of some members of the DGC at the muscle cell surface (Ohlendieck and Campbell, 1991). Furthermore, severe childhood autosomal recessive muscular dystrophy has been linked to mutations affecting the adhalin gene (Roberds et al., 1994), suggesting that mutations affecting the transmembrane linkage between the ECM and the muscle cytoskeleton through the DGC will translate into a severe myopathy. In agreement with this hypothesis, muscular dystrophy in the *dy/dy* mouse and a form of congenital muscular dystrophy in humans results from a deficiency in the ECM protein merosin (Tomé et al., 1994; Sunada et al., 1994), a ligand for the DGC in muscle.

About 30% of DMD patients suffer to varying degrees from mental retardation (Hyser and Mendell, 1988), and some also show evidence of an abnormal electroretinogram (ERG) indicative of defective synaptic transmission in the retina outer plexiform layer (OPL; Pillers et al., 1993). Dystrophin has been localized to postsynaptic regions in brain (Lidov et al., 1990) and retina (Schmitz et al., 1993) raising the possibility of a similar synaptic localization of α- and β-DG in the central nervous system (CNS). We therefore examined the expression of DG in the retina where synaptic layers are readily distinguishable, and compared it to that of dystrophin, DRP and one of its ECM ligands, laminin.

We find that DG is coexpressed with dystrophin and DRP in the OPL where photoreceptors, bipolar neurons, and horizontal cells synapse onto each other. It also co-distributes with DRP and laminin around blood vessels and in the inner limiting membrane. In order to address the question of an interaction between dystrophin and DG in the CNS, we also studied the retina of the *mdx* mouse, a DMD animal model where the higher molecular weight dystrophin isoform (427 kDa) is not expressed. The distribution of α- and β-DG in the mdx retina is identical to that in the wild type mouse. This is consistent with a recent report (D'Souza et al., 1995) showing that *mdx* mice, unlike DMD patients, have a normal ERG (see Discussion). Furthermore, using an antibody directed against the carboxyl-terminus of dystrophin, we find immunoreactivity in the *mdx* mouse retina identical to that in the wild type mouse, confirming the presence of dystrophin isoforms in the mdx retina.

MATERIALS AND METHODS

Animals and Antibodies

Adult male C57BL/10ScSn wild type mice and C57BL/10ScSn-mdx/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). The polyclonal rabbit anti-laminin antibody was made in our laboratory to purified mouse Engelebreth-Holme-Swarm sarcoma laminin and recognizes laminin A chain as well as B1 and B2 chains. The polyclonal rabbit anti-dystrophin antibody was a generous gift from Dr. P. Holland at the Montreal Neurological Institute and was raised and affinity-purified against a peptide encompassing the last 17 amino acids of human dystrophin. This antibody does not cross-react with DRP (Dr. P. Holland, personal communication). The polyclonal rabbit anti-DRP antibody was a gift from Dr. L.M. Kunkel at Howard Hughes Medical Institute in Boston. It was raised against a fusion protein derived from the DRP cDNA and was "Affigel Blue" purified. This antibody has been previously characterized and shown not to cross-react with dystrophin (Khurana et al., 1990). The two polyclonal antibodies to DG were raised and affinity-purified against two fusion proteins derived from different regions of DG transcript. The anti-fusion protein D antibody (anti-FPD) recognizes the core protein of α-DG exclusively, while the anti-fusion protein B antibody (anti-FPB) recognizes both α- and β-DG. Both antibodies have been previously characterized (Ibraghimov-Beskrovnaya et al., 1992).

Immunohistochemistry

For immunohistochemistry with sheep anti-FP B, sheep anti-FP D, and rabbit anti-laminin antibodies, tissues were fixed for 2 hours in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C. The cornea of the eye was cut prior to fixation in order to facilitate the penetration of the paraformaldehyde. Tissues were washed in ice-cold phosphate-buffered saline (PBS), infused overnight in 10% sucrose at 4°C, and then frozen in 2-methylbutane cooled to −60°C in liquid nitrogen. Sections of the eye were cut in a temporal to nasal direction in the vicinity of the optic disc while skeletal muscle tissue was cut transversally. Eight-μm-thick cryosections were mounted on Probe-On Plus slides (Fisher Scientific, Ottawa, ON) and used the same day for immunohistochemistry. Sections were postfixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100.

For immunohistochemistry with rabbit anti-dystrophin and rabbit anti-DRP antibodies, tissues were fresh-frozen in 2-methylbutane cooled in liquid nitrogen. Sections were then fixed and permeabilized in cold acetone (−20°C). After this treatment the inner limiting membrane was often torn or remained attached to the lens.

Autofluorescence in the inner photoreceptor segment was significantly decreased by treatment of the retinal sections with 0.3% (w/v) sodium borohydride for 2 min. All sections were then blocked with 10% horse serum in PBS, incubated with the primary antibody (anti-FP B at 1:5; anti-FP D undiluted; anti-laminin at 1:50; anti-dystrophin at 1:20; anti-DRP at 1:250), then

with the appropriate secondary antibody conjugated to biotin (rabbit anti-sheep at 1:200; goat anti-rabbit at 1:200), followed by treatment with streptavidin conjugated to fluorescein (1:200). In control sections the primary antibody was either omitted or replaced with preimmune serum (only for the anti-DRP antibody). As an additional control, some sections were only treated with streptavidin-fluorescein. Sections were mounted in Immunofluor mounting medium (ICN, Costa Mesa, CA), viewed under a Zeiss fluorescence microscope, and photographed with a 400 ASA T-MAX Kodak film.

Northern Blot Analysis

Total RNA from cerebellum, olfactory bulb, cerebral cortex, basal ganglia, hippocampus, brainstem, whole eye (without extraocular muscles) and skeletal muscle from wild type and *mdx* adult mice was prepared using the one-step guanidium isothiocyanate technique (Chomczynski and Sacchi, 1987). Northern blot analysis was carried out exactly as described in Gee et al. (1994). The probe, a 400 bp BamHI/SacI fragment derived from the mouse α-DG cDNA, was labelled with [α-^{32}P]-CTP (3,000 Ci/nmol; Amersham, Oakville, ON) using the random primer technique, then purified by gel filtration (Nuc-Trap column, Stratagene, Aurora, ON).

In Situ Hybridization

A 400 bp BamHI/SacI fragment derived from the mouse α-DG cDNA inserted in a pBluescript II SK+ phagemid (Stratagene) was labelled with [^{35}S]-UTP (1,000 Ci/mmol; NEN, Mississauga, ON) via in vitro transcription (Sambrook et al., 1989). Sense and antisense probes were generated by transcription with either T7 or T3 RNA polymerase (Promega, Madison, WI). Probes were further purified by gel filtration; 10^7 counts were used per slide.

Eyes from wild type and *mdx* mice were fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer for 5 hours, washed in RNase-free PBS, and infused overnight in 10% RNase-free ice-cold sucrose in PBS. The lens was removed and the tissues were then frozen in 2-methylbutane cooled to −60°C in liquid nitrogen. Eight-μm-thick sections were mounted on Probe-On Plus slides (Fisher Scientific), briefly air-dried, then stored at −80°C until needed for analysis. Slides were lyophilized, rinsed in PBS, acetylated in 100 mM triethanolamine (pH 7.0), 0.25% acetic anhydride, rinsed in PBS, and prehybridized in hybridization solution (2× SSC [0.3 M NaCl, 35 mM sodium citrate, pH 7.0], 50% formamide, 1 mM DTT, 250 μg/ml transfer RNA, 5 mM EDTA [pH 8.0], 0.2% SDS, 250 μg/ml, 1X Denhardt's solution) for 1 hour at 42°C. Sections were subsequently hybridized overnight at 42°C in hybridization solution containing either the sense or antisense [^{35}S]-labelled DG-specific probe, washed at 45°C twice in 4× SSC, 1 mM DTT, then twice in 4× SSC alone. Excess probe was digested with 50 μg/ml ribonuclease A in 0.5 M NaCl, 10 mM Tris, 5 mM EDTA (pH 8.0) for 30 min at 37°C. Slides were sequentially washed for 30 min at 65°C twice in 2× SSC, once in 1× SSC, and once in 0.1× SSC. They were dehydrated through graded ethanol to remove excess salt, air-dried, dipped in Kodak NBT2 autoradiography emulsion, and developed 2 weeks later. At this stage, sections were counterstained with neutral red, permanently mounted in Permount (Fisher), and photographed in darkfield under a 25× objective using a technical Pan Kodak film.

Western Blot Analysis

Upon dissection, tissues were extracted in 1× reducing sample buffer (67.5 mM Tris-HCl [pH 6.8], 20% glycerol, 15% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) as described by Lederfein et al. (1992). One ml of reducing sample buffer was used per 0.1 g of wet tissue. Muscle tissue was homogenized in a Polytron homogenizer, while neural tissues and liver were manually homogenized using Dounce homogenizers. Ten μl of muscle, cerebellum and liver extracts, and 30 μl of neural retina extracts were loaded per lane on 5% and 10% SDS-polyacrylamide gels (Laemmli, 1970) and run at 150 V for 1 hour. Proteins were subsequently transferred to nitrocellulose for 75 min at 100 V (Towbin et al., 1979). Blots were blocked for 2 hours with 5% skim milk, 3% bovine serum albumin (BSA) in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20, and subsequently incubated for 1 hour with anti-dystrophin antibody at a dilution of 1:500, washed for 1 hour, incubated in the presence of a goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase, and extensively washed for 2 hours. The specific signal was then visualized on a Kodak X-ray film using enhanced chemiluminescence (NEN, Mississauga, ON).

RESULTS

DG mRNA Expression in the CNS

In order to map DG expression within the adult wild type and *mdx* mouse CNS, we probed Northern blots of RNA from microdissected brain regions and from whole eyes with a radiolabelled mouse DG cDNA probe. A single band of about 5.8 kb, equivalent in size to the mRNA for DG in skeletal muscle although less abundant (Fig. 1; Ibraghimov-Beskrovnaya et al., 1992), is detected in RNA from all CNS regions and eye. DG mRNA is expressed at similar levels in brainstem, cerebellum, basal ganglia, cerebral cortex, olfactory bulb, and eye, but is somewhat less abundant in the hippocam-

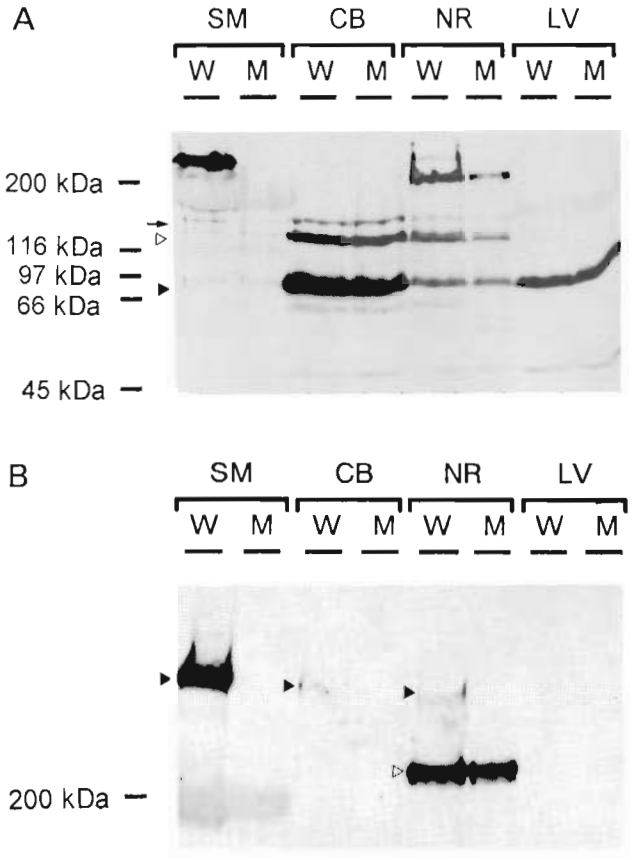

Fig. 6. Dystrophin isoforms are expressed in both wild type and mdx nonmuscle tissues. Western blot analysis of crude protein extracts from skeletal muscle (SM), cerebellum (CB), neural retina (NR), and liver (LV) from wild type (W) and *mdx* (M) mouse with an anti-dystrophin antibody. **A:** Ten percent polyacrylamide gel showing the expression pattern of lower molecular weight dystrophin isoforms. An immunoreactive protein of 71 kDa (filled arrowhead) is present in all nonmuscle tissues of both wild type and *mdx* mice. One hundred fifty (arrow) and 125 (open arrowhead) kDa dystrophin isoforms are only expressed in neural tissues. The fainter immunoreactivity in the mdx retina compared to wild type is due to a difference in the amount of protein loaded. **B:** Five percent polyacrylamide gel showing high molecular weight dystrophin. Full length dystrophin of 427 kDa (filled arrowheads) is expressed in skeletal muscle, cerebellum and neural retina from wild type mice only. A ~220 kDa (open arrowhead) immunoreactive band is detected exclusively in neural retina. Molecular weight markers are indicated in the left margins.

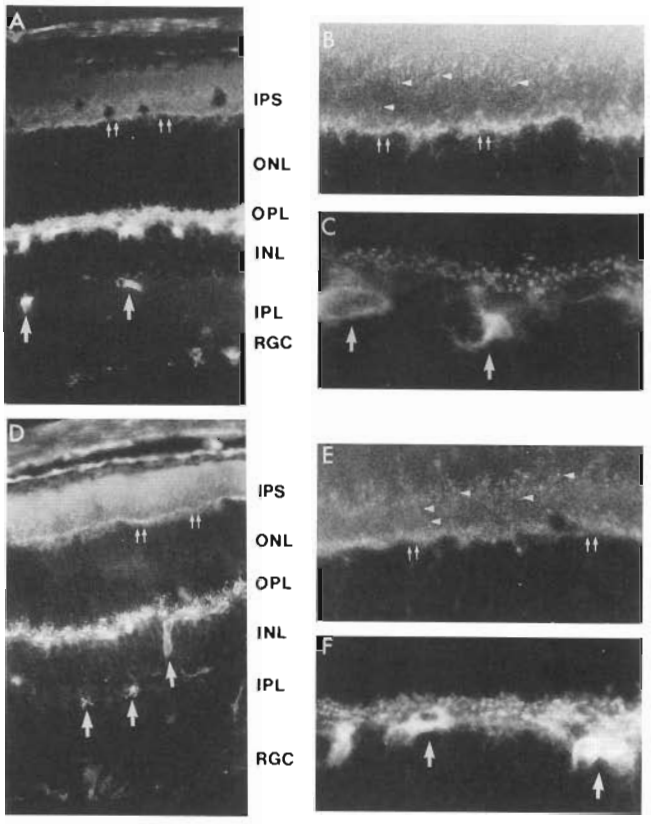

Fig. 7. DRP is expressed in but not restricted to the OPL. **A,D:** DRP immunoreactivity in wild type and mdx retina, respectively, is present in the OPL, and around blood vessels (large arrows) where α- and β-DG are also expressed, as well as in the IPS and OLM (small arrows). See abbreviations in Figures 2 and 3. Magnification 320×. **B,E:** Enlargement of the IPS region from wild type and mdx retina, respectively, showing labelling of the OLM (arrows) and the punctate DRP immunoreactivity (arrowheads) in the IPS. Magnification 1,280×. **C,F:** Enlargement of the OPL from wild type and mdx retina, respectively, highlighting the punctate pattern of DRP immunoreactivity similar to that of dystrophin. Blood vessels are indicated by arrows. Magnification 1,280×.

distribution in the retina. Using a polyclonal antibody to laminin that recognizes the A, B1 and B2 chains, we confirmed laminin's restricted expression in the adult mouse retina to the basement membrane around blood vessels and at the ILM where it colocalizes with both DRP, and α- and β-DG (Fig. 8A,B). However, laminin was absent from both the OPL and the IPS. No differences in terms of intensity of immunoreactivity or general appearance were detectable between wild type and mdx retina. Like DRP and α- and β-DG, laminin was also detected in the sclera (Fig. 8B, top), ciliary muscle and ciliary processes (not shown).

DISCUSSION

We have shown that two members of the DGC identified in muscle, α- and β-DG, are expressed in the adult CNS and colocalize at a synaptic region with dystrophin and DRP, two potential cytoskeletal ligands. In

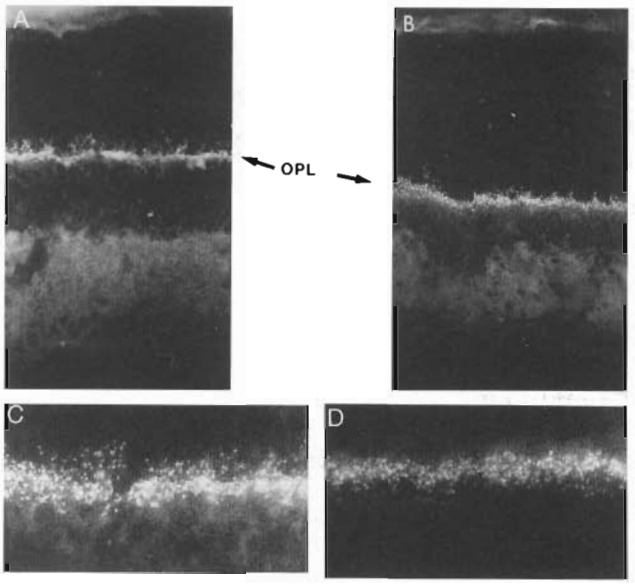

Fig. 4. Dystrophin is expressed in the OPL of both wild type and mdx retina. **A,B:** Retina sections from wild type and *mdx* mouse, respectively, show dystrophin immunoreactivity in the OPL, indicating that dystrophin isoforms are expressed in the mdx retina. The diffuse faint labelling in other layers is not specific and is also present when the immune serum is omitted. Magnification 320×. **C,D:** Enlargements of the OPL region from wild type and mdx retina, respectively, showing the distinct immunoreactive pattern of dystrophin. Magnification 1,280×.

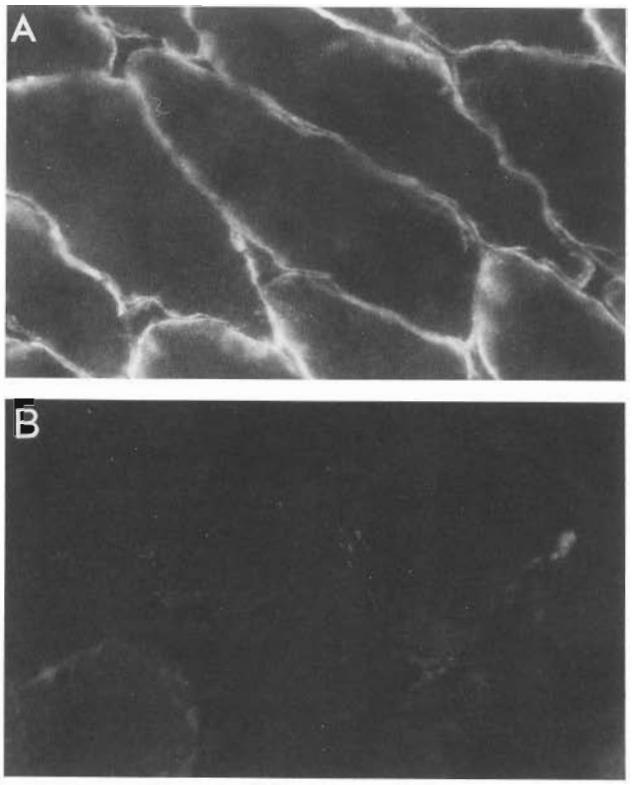

Fig. 5. Dystrophin is not expressed in mdx muscle. **A:** Skeletal muscle sections from wild type mouse labelled with the anti-dystrophin antibody show immunoreactivity at the level of the sarcolemma of each muscle fiber. **B:** Skeletal muscle sections from *mdx* mouse show no immunoreactivity for dystrophin. Magnification 544×.

ilar results upon blotting with the anti-dystrophin antibody (Fig. 6A). The 150, 125 and 71 kDa major dystrophin isoforms are all present (Fig. 6A) with the addition of a 220 kDa protein not expressed in cerebellum nor skeletal muscle (Fig. 6B). Full length dystrophin was only expressed in wild type neural retina (Fig. 6B). Since dystrophin isoforms have previously been reported to be expressed in liver using a variety of antibodies to different domains of dystrophin (Byers et al., 1993; Lederfein et al., 1992; Fabbrizio et al., 1994), we looked at liver as an additional control tissue and found abundant expression of a 71 kDa dystrophin isoform (Fig. 6A). No full length dytrophin was detected in liver even after prolonged exposures (Fig. 6B). It would therefore seem that dystrophin isoforms are indeed expressed in mdx non-muscle tissues, including the neural retina where they all colocalize to the OPL (Fig. 4).

Dystrophin-Related Protein and Laminin

Like dystrophin, DRP can interact with members of the DGC in muscle and is present at myoneural (Ohlendieck et al., 1991b; Khurana et al., 1991; Thi Man et al., 1991) and CNS synapses (Kamakura et al., 1994). Consistent with this, polyclonal antibodies for DRP strongly label both wild type and mdx OPL (Fig. 7A,C,D,F) in a punctate pattern similar to that of dystrophin (Fig. 4). Immunoreactivity is also detected in nonsynaptic regions such as the inner limiting membrane (ILM, not shown), at the level of the IPS and outer limiting membrane (OLM), as well as around blood vessels (Fig. 7A,D). A diffuse labelling is observed at the OLM, while a fine punctate immunoreactive pattern is discernable in the IPS (Fig. 7B,E). The latter was only observed with antibodies to DRP and vimentin (data not shown), an intermediate filament expressed by Müller glia (Reichenbach et al., 1991). The strong immunoreactivity around blood vessels and the previous report that DRP is expressed at astrocytic endfeet in the CNS (Khurana et al., 1992), suggest that Müller glia may express DRP. Outside the neural retina, DRP immunoreactivity was detected in the sclera (Fig. 7A,B, top), ciliary muscles and ciliary processes (not shown). Control sections where the primary antibody was replaced with preimmune serum showed no immunoreactivity.

Since laminin is a ligand for α-DG, we looked at its

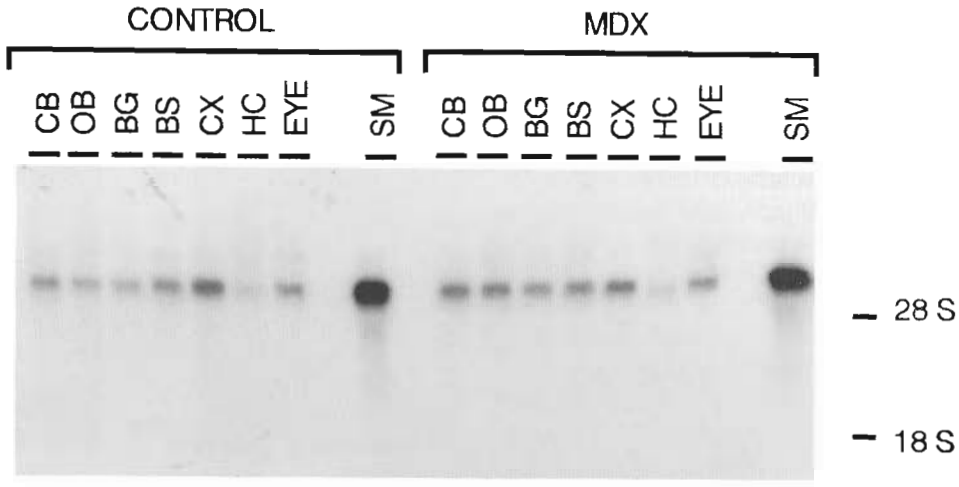

Fig. 1. Dystroglycan mRNA expression in control (wild type) and mdx CNS. Northern blot analysis of total RNA reveals no differences in DG mRNA expression levels between control and mdx mouse neural and skeletal muscle tissues. A single 5.8 kb transcript is detected in all tissues tested. The positions of 18S and 28S ribosomal RNA are shown on the left. BG, basal ganglia; BS, brainstem; CB, cerebellum; CX, cerebral cortex; HC, hippocampus; OB, olfactory bulb; SM, skeletal muscle.

pus. The expression of DG mRNA is essentially the same between wild type and *mdx* mice for these CNS regions as well as for muscle and eye. As shown previously (Ibraghimov-Beskrovnaya et al., 1992), the *mdx* mutation does not affect the steady state levels of DG mRNA in skeletal muscle; DG expression throughout the CNS is similarly unaffected (Fig. 1).

α- and β-Dystroglycan in the Retina

Because of its possible involvement in synaptic structure (Gee et al., 1994), DG was localized immunohistochemically in the retina where synaptic regions are readily distinguishable. Immunohistochemistry was performed on retinal sections with antibodies recognizing either α-DG alone (anti-FPD), or both α- and β-DG (anti-FPB). In both wild type and mdx retina, the strongest immunoreactivity with either antibodies is found around blood vessels, and the inner limiting membrane (ILM; Fig. 2A,B). Weaker but reproducible immunoreactivity is present in the OPL where photoreceptors, bipolar cells and horizontal neurons synapse onto each other. The OPL is uniformly labelled with an underlying punctate immunoreactive pattern (Fig. 2C). Other structures outside the neural retina were also found to be immunoreactive. These include the sclera, the ciliary muscles and ciliary processes (not shown). Control sections where the primary antibody was omitted showed no immunoreactivity.

In order to confirm the presence of α- and β-DG in the OPL and in an attempt to determine which cells are responsible for it, we performed in situ hybridization on retinal sections using antisense and sense RNA probes

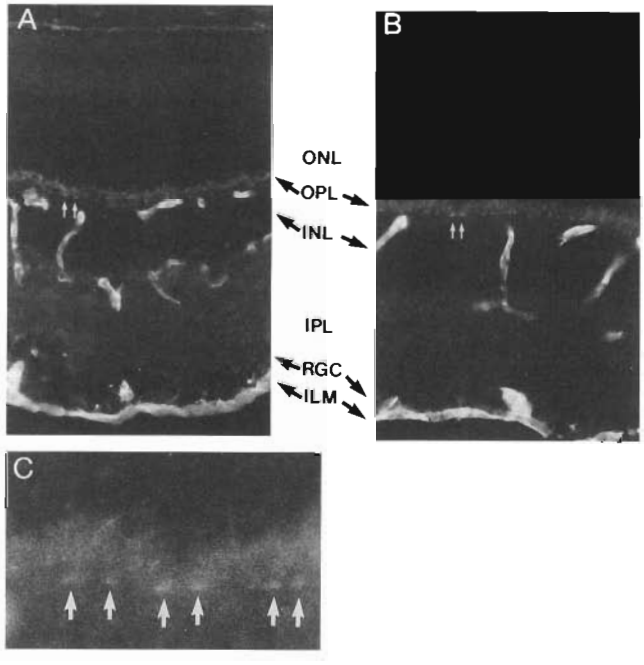

Fig. 2. Distribution of α- and β-DG in wild type and mdx retina. Immunohistochemistry with anti-FPB antibody to both α- and β-DG reveals their presence in the OPL, the ILM and around blood vessels in both wild type (**A**) and mdx (**B**) retina. Immunoreactivity in the OPL is uniform with an underlying punctate pattern (arrows). ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC, retinal ganglion cell layer. Magnification 352×. **C:** Enlargement of the OPL region of a wild type retina highlighting the punctate pattern of α- and β-DG immunoreactivity in that layer (arrows). Magnification 1,122×.

derived from the 400 bp BamHI/SacI fragment previously used for Northern blot analysis. Wild type and mdx retinal sections hybridized with the antisense probe (Fig. 3A,C) show a concentration of silver grains over the photoreceptor inner segment (IPS) where most of the protein synthesis occurs (Rodieck, 1973), as well as the inner nuclear layer (INL) and individual retinal ganglion cells (RGC). Retinal sections hybridized with the sense RNA probe (control) have few silver grains (Fig. 3B,D). The presence of DG mRNA in photoreceptors suggests that these are one of the neuronal populations contributing to the presence of α- and β-DG immunoreactivity in the OPL. The labelling over RGCs is in agreement with the presence of α- and β-DG immunoreactivity in the optic nerve (unpublished observation).

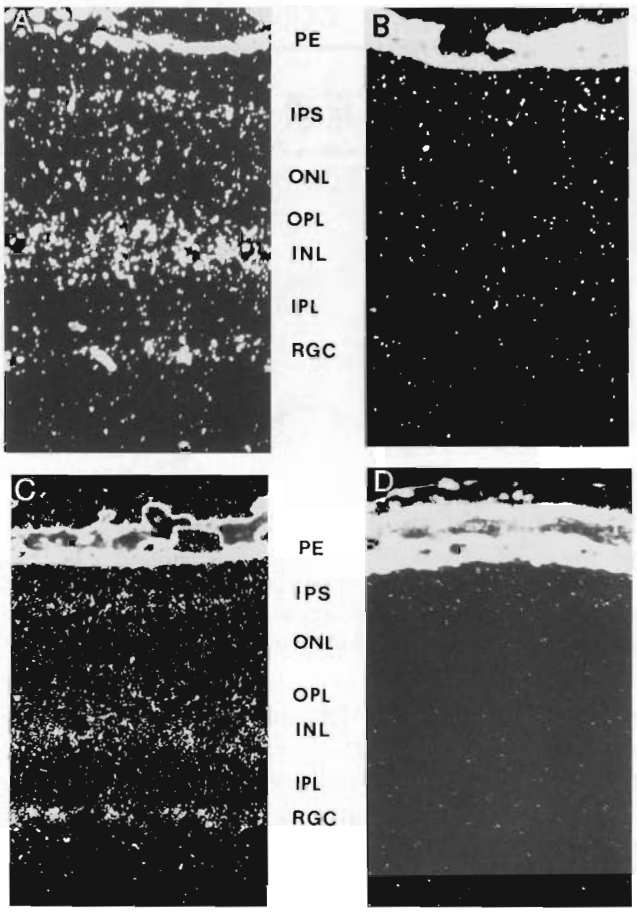

Fig. 3. DG is expressed in all nuclear layers in wild type and mdx retina. **A,C:** Darkfield view of control and mdx retina sections, respectively, hybridized to [^{35}S]-labelled DG-specific antisense RNA probe. Silver grains are concentrated above RGC, the INL, and the photoreceptor inner segment. Deposition above both plexiform layers is comparable to background. **B,D:** Darkfield view of wild type and mdx retina sections, respectively, hybridized to [^{35}S]-labelled DG-specific sense RNA probe as a control for the specificity of the signal in A and C. See abbreviations in Figure 2; IPS, inner photoreceptor segment; PE, pigmented epithelium. Magnification 283×.

Dystrophin and Dystrophin Isoforms

Using a polyclonal antibody directed against the last 17 amino acids of full length dystrophin (427 kDa), we confirmed (Fig. 4A,C) the restricted and punctate expression of dystrophin in the OPL of wild type mice reported by Miike et al. (1989). The faint staining in the inner plexiform layer (IPL) and IPS is also present in control sections where the primary and secondary antibodies were omitted and is probably a fixation artifact. Somewhat surprisingly, a similar pattern of immunoreactivity was found in the OPL of *mdx* mice (Fig. 4B,D). In order to confirm that the antibody does not cross-react with DRP, we looked at the distribution of dystrophin in transverse skeletal muscle sections from all mice whose retinas were used for immunocytochemistry. All wild type mice showed dystrophin immunoreactivity in all their muscle fibers at the level of the sarcolemma (Fig. 5A). The same antibody does not show any immunoreactivity in skeletal muscle sections from *mdx* mice (Fig. 5B) and is apparently specific for dystrophin with no detectable cross-reactivity with DRP which is expressed in mdx muscle (Thi Man et al., 1991; Ohlendieck et al., 1991b; Khurana et al., 1991).

To determine whether dystrophin isoforms are recognized by the antibody in wild type and mdx retina, we performed Western blot analysis of skeletal muscle, cerebellum, and neural retina (Fig. 6). Protein extracts were run on 10% (Fig. 6A) and 5% (Fig. 6B) polyacrylamide gels in order to resolve lower and higher molecular weight dystrophin isoforms, respectively. In wild type skeletal muscle, the antibody recognized a single band above the 200 kDa molecular weight marker likely to correspond to full length dystrophin of 427 kDa (Fig. 6A,B). The antibody did not recognize any proteins in mdx skeletal muscle (Fig. 6A,B), and did not cross-react with DRP (420 kDa) in mdx or wild type skeletal muscle. Fainter bands are also seen with the secondary antibody alone and correspond to abundant proteins present in the skeletal muscle extracts. In extracts from wild type cerebellum, a CNS region particularly rich in dystrophin (Lidov et al., 1990; Gorecki et al., 1991), the antibody identified three major bands with estimated molecular weights of 150, 125 and 71 kDa (Fig. 6A). A fainter band with a relative mobility similar to full length dystrophin (427 kDa) detected in muscle was also present in wild type cerebellum (Fig. 6B). The 150, 125 and 71 kDa dystrophin isoforms were also present in the mdx cerebellum (Fig. 6A), while the less abundant 427 kDa dystrophin was undetectable (Fig. 6B). Neural retina protein extracts from wild type and *mdx* mice gave sim-

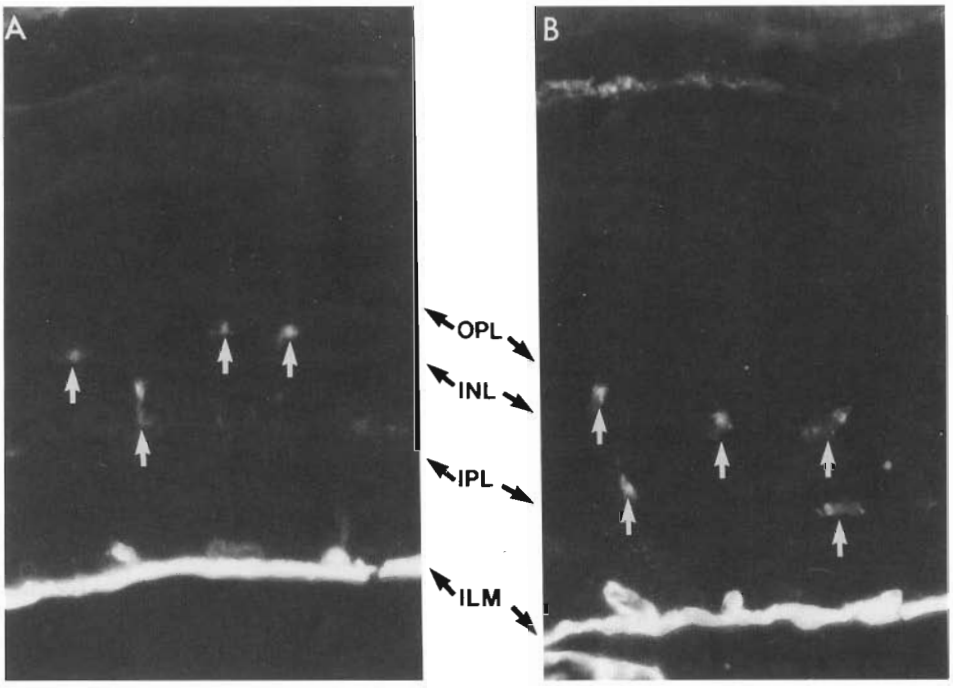

Fig. 8. Laminin only partially colocalizes with α-DG in the retina. Laminin immunoreactivity is localized to the ILM and blood vessels (arrows) in both wild type (**A**) and mdx (**B**) retina. However, it is absent from the OPL where α- and β-DG are expressed. Magnification 450×.

light of the postulated functional role played by the DGC in the organization and stabilization of the neuromuscular junction (Gee et al., 1994; Campanelli et al., 1994; Bowe et al., 1994), the coexpression of α-DG, β-DG, dystrophin, and DRP in a synaptic layer in the retina has important implications for their role in CNS synaptogenesis.

While all members of the DGC are present in skeletal and cardiac muscle (Ohlendieck et al., 1991a; Klietsch et al., 1993), only syntrophins and α- and β-DG have so far been detected in nonmuscle tissues, including CNS (Mizuno et al., 1993; Adams et al., 1993). It has been postulated that α- and β-DG form a subcomplex in muscle (Yoshida et al., 1994) and are potentially capable of mediating interactions with both dystrophin and DRP as well as laminin and agrin in the ECM (Ervasti and Campbell, 1993; Gee et al., 1994; Suzuki et al., 1994; Yamada et al., 1994). Consistent with this, affinity purification of agrin-binding proteins from Torpedo electric organ membranes yields only α- and β-DG complexes (Bowe et al., 1994). Thus, this complex might predominate in the CNS and other nonmuscle tissues.

We detect a single DG transcript of 5.8 kb whose wide distribution in the CNS parallels that reported for dystrophin (Lidov et al., 1990; Gorecki et al., 1991) and DRP (Kamakura et al., 1994). Indeed, immunohistochemistry on brain sections has confirmed the presence of α- and β-DG at the level of the glia limitans, meninges, and around blood vessels (Montanaro and Carbonetto, unpublished results), a distribution strongly reminiscent of that described for DRP (Khurana et al., 1992; Kamakura et al., 1994). Furthermore, as we previously reported (Montanaro et al., 1993), we find that in the retina α- and β-DG both codistribute with DRP and dystrophin in a synaptic layer, the OPL, as well as with DRP and laminin around blood vessels and at the retinal ILM. We have never seen expression of α- and β-DG in regions where neither dystrophin nor DRP were present, a correlation also reported by Gorecki et al. (1994) at the RNA level in brain. In addition, we have identified two neuronal cell types, photoreceptors and RGCs, that express DG mRNA as determined by in situ hybridization. Expression of DG mRNA in the INL could not be attributed with absolute certainty to any particular population of cells. The expression of DG mRNA by photoreceptors, in conjunction with the absence of α- and β-DG immunoreactivity in retinal layers distal to the OPL (INL, inner and outer photoreceptor segments), suggests that α- and β-DG are localized to the photoreceptor synaptic terminals in the OPL. RGC, on the other hand, might target α- and β-DG to their axonal compartment in apposition with the laminin-rich ILM. It would be of interest to determine whether RGC also have α- and β-DG at their synaptic terminals in the cortical areas to which they project.

α-DG has been characterized as a high affinity laminin receptor in both muscle and nonmuscle tissues (Douville et al., 1988; Gee et al., 1993; Yamada et al., 1994; Ervasti and Campbell, 1993), and has been shown to colocalize with laminin in skeletal and cardiac muscle, as well as peripheral nerve (Klietsch et al., 1993; Yamada et al., 1994). Consistent with its hypothesized function as a laminin receptor, we find that α-DG colocalizes with laminin to the ILM and around blood vessels in the neural retina. However, this correlation does not extend to the OPL where laminin immunoreactivity is not detected, raising the question of the identity of the ECM molecule, if any, that interacts with α-DG in that layer. α-DG has been shown to recognize a specific region of laminin A chain containing several G-repeat domains (Gee et al., 1993). Homologous structural units are found in several other proteins, including the laminin A chain isoform, merosin (Vuolteenaho et al., 1994), the synapse organizing molecule, agrin (Rupp et al., 1991), and members of a family of presynaptic proteins known as neurexins (Ushkaryov et al., 1992). While both merosin (Tian et al., 1994) and neurexins (Ushkaryov et al., 1992) have been localized to synaptic sites in brain regions also expressing DG, their distribution in the neural retina has not yet been reported. In addition, the ability of neurexins to interact with α-DG has not yet been assessed. Agrin, on the other hand, is expressed by RGC (Ma et al., 1994) and has been immunohistochemically localized to the OPL, the optic fiber layer, and the ILM (Kröger et al., 1994) where α- and β-DG are also present. Since agrin binding to α-DG in muscle has been postulated to be essential for the reorganization of postsynaptic elements, such as acetylcholine receptors (Gee et al., 1994), agrin is a potential ligand for α-DG in the OPL, possibly hinting at a role for α- and β-DG in CNS synaptogenesis.

In muscle, the DGC is tightly associated with dystrophin, and the absence of the latter induces a significant reduction in the levels of α- and β-DG at the cell surface without affecting the levels of DG mRNA in the *mdx* mouse (Ibraghimov-Beskrovnaya et al., 1992). Aside from the severe myopathy, a majority of DMD patients display some degree of cognitive impairment (Hyser and Mendell, 1988) and some have been reported to have an abnormal b-wave in their ERG symptomatic of defective synaptic transmission in the OPL (Pillers et al., 1993). We attempted to make use of an animal model for DMD, the *mdx* mouse, to ascertain whether the mutation in the dystrophin gene affected the expression of DG, providing further evidence for a physiologically relevant link between dystrophin and α- and β-DG in the CNS. However, we were unable to detect any differences in the expression of DG in the retina at either the mRNA or protein level. This could be due to α- and β-DG interacting primarily, but not necessarily exclusively, with DRP rather than dystrophin in the OPL. In at least one other synaptic region, the neuromuscular junction, α- and β-DG have been found to be unaffected by the *mdx* mutation due to the interaction of the DGC with DRP. Moreover, we find DG mRNA expression in photoreceptors while dystrophin has been reported to be present at the postsynaptic terminals of bipolar and horizontal cells (Schmitz et al., 1993). These observations would imply that α- and β-DG are presynaptic while dystrophin is postsynaptic. However, the presence of DG mRNA in the INL does not exclude the possibility that α- and β-DG are coexpressed with dystrophin in horizontal and bipolar cell dendrites. Therefore α- and β-DG could be both pre- and postsynaptically expressed in the retina. It would be of interest to determine whether DRP is expressed presynaptically.

Alternatively, dystrophin isoforms containing the C-terminal sequences necessary for the interaction with β-DG could be expressed in the mdx retina. In addition to full length dystrophin present exclusively in wild type mice, we detected the presence of five dystrophin-immunoreactive proteins of estimated molecular weights 220, 150, 125 and 71 kDa in both wild type and mdx neural retina. Although we cannot rule out the possibility that some of these dystrophin-like immunoreactive proteins might be cross-reactive proteins, dystrophin isoforms with similar molecular weights have been previously reported to be expressed in nonmuscle tissues using a variety of anti-dystrophin antibodies (Hugnot et al., 1992; Lederfein et al., 1992; Kim et al., 1992; Byers et al., 1993; Mizuno et al., 1993; Fabbrizio et al., 1994; D'Souza et al., 1995). Furthermore, expression of Dp71 and a novel Dp260 has been recently reported in wild type and mdx retina (D'Souza et al., 1995). A 260 kDa dystrophin isoform present exclusively in neural retina has also been previously reported to be expressed in humans (Pillers et al., 1993, Schmitz et al., 1993) and could correspond to the estimated 220 kDa protein we detect in both wild type and mdx retina.

Several dystrophin isoforms, lacking progressively larger portions of the amino-terminal half of full length dystrophin, appear to be coexpressed in a single retinal synaptic layer and are all potentially capable of interacting with α- and β-DG. Although the reason for this variety of dystrophin isoforms in nonmuscle tissues is unclear at present, we could speculate that they might serve different functions at a single synaptic terminal. It should also be pointed out that in muscle, the amino-terminus of dystrophin plays an essential structural role as evidenced by the inability of Dp71 to prevent dystrophy while restoring DGC expression at the cell surface (Cox et al., 1994; Greenberg et al., 1994). However, a recent report by D'Souza et al. (1995) suggests that lack

of Dp260 rather than full length dystrophin is responsible for the abnormal ERG in both DMD and the *mdx*^{CV3} mouse, whereas its expression in the *mdx* mouse accounts for its normal ERG. This novel dystrophin isoform has the cysteine-rich domain that interacts with β-DG which could be responsible for the unaltered distribution of α- and β-DG that we observe in mdx retina. Therefore, if α- and β-DG do interact with dystrophin isoforms in the CNS, this complex might have functions quite different from those proposed in muscle. In this regard, the *mdx*^{CV3} mutant (Pillers et al., 1995; D'Souza et al., 1995) with abnormal expression of several non-muscle dystrophin isoforms and an abnormal ERG could provide an interesting animal model to address these questions.

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NOTE ADDED IN PROOF

Recently Smalheiser and Kim (J Biol Chem 270: 15425–15433, 1995) reported the immunolocalization of α-dystroglycan to rat cerebellar neurons, particularly within the dendrites of Purkinje neurons. This finding further supports a synapse specific function for α-dystroglycan in the CNS.

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