Dystroglycan in the Cerebellum is a Laminin α2-chain Binding Protein at the Glial–Vascular Interface and is Expressed in Purkinje cells

M. Tian[1], C. Jacobson[2], S. H. Gee[2], K. P. Campbell[3], S. Carbonetto[2] and M. Jucker[1]

[1]Gerontology Research Center, National Institute on Aging, National Institute of Health, Baltimore, MD 21224, USA
[2]Center for Research in Neurosciences, Montreal General Hospital Research Institute, McGill University, Montreal, H3G 1A4, Canada
[3]Howard Hughes Medical Institute, and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242, USA

Abstract

Dystroglycan is a core component of the dystrophin receptor complex in skeletal muscle which links the extracellular matrix to the muscle cytoskeleton. Dystrophin, dystrophin-related protein (DRP, utrophin) and dystroglycan are present not only in muscles but also in the brain. Dystrophin is expressed in certain neuronal populations while DRP is associated with perivascular astrocytes. To gain insights into the function and molecular interactions of dystroglycan in the brain, we examined the localization of α- and β-dystroglycan at the cellular and subcellular levels in the rat cerebellum. In blood vessels, we find α-dystroglycan associated with the laminin α2-chain-rich parenchymal vascular basement membrane and β-dystroglycan associated with the endfeet of perivascular astrocytes. We also show that α-dystroglycan purified from the brain binds α2-chain-containing laminin-2. These observations suggest a dystroglycan-mediated linkage between DRP in perivascular astrocytic endfeet and laminin-2 in the parenchymal basement membrane similar to that described in skeletal muscle. This linkage of the astrocytic endfeet to the vascular basement membrane is likely to be important for blood vessel formation and stabilization and for maintaining the integrity of the blood–brain barrier. In addition to blood vessel labelling, we show that α-dystroglycan in the rat cerebellum is associated with the surface of Purkinje cell bodies, dendrites and dendritic spines. Dystrophin has previously been localized to the inner surface of the plasma membrane of Purkinje cells and is enriched at postsynaptic sites. Thus, the present results also support the hypothesis that dystrophin interacts with dystroglycan in cerebellar Purkinje neurons.

Introduction

Duchenne muscular dystrophy is a lethal muscle disorder resulting from a mutation in the gene for dystrophin which leads to the absence of this subsarcolemmal cytoskeletal protein (Ahn and Kunkel, 1993). In muscle tissue, dystrophin is associated with a complex of cell surface proteins which span the plasma membrane linking the extracellular matrix proteins laminin and agrin to the muscle cytoskeleton (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993; Gee et al., 1993, 1994; Bowe et al., 1994; Campanelli et al., 1994). Dystrophin-related protein (DRP or utrophin) is an autosomally encoded dystrophin homologue and is also localized to the subsarcolemmal cytoskeleton where it interacts with the dystrophin receptor complex (Matsumara et al., 1992; Ahn and Kunkel, 1993).

Dystrophin and DRP are present not only in muscles but also in the brain. Dystrophin is expressed in specific subsets of neurons and is enriched at postsynaptic sites (Gorecki et al., 1992; Lidov et al., 1990, 1993). Although its function in the brain is not understood, the lack of dystrophin in the brain of Duchenne muscular dystrophy

patients might be the cause of their cognitive deficits (Lidov et al., 1990; Billard et al., 1992). In contrast to the neuronal localization of dystrophin, DRP in the brain is mainly associated with the cerebral blood vessels, choroid plexus, ependymal lining and pia mater (Khurana et al., 1992). Ultrastructural analysis has localized DRP to the inner face of the plasma membrane of perivascular astrocytic foot processes (Khurana et al., 1992). More recently, truncated forms of dystrophin and DRP derived from alternative promoters have also been described in the brain (Lederlein et al., 1992; Schofield et al., 1994; Blake et al., 1995).

α-Dystroglycan (α-DG) and β-dystroglycan (β-DG), two core components of the dystrophin receptor complex in skeletal muscle, are also expressed in the brain (Smalheiser and Schwartz, 1987; Douville et al., 1988; Ibraghimov-Beskrovnaya et al., 1992; Gee et al., 1993; Smalheiser and Kim, 1995). The dystroglycan transcript is expressed in neurons of the hippocampus, olfactory bulb, thalamus, retina and cerebellum (Gorecki et al., 1994; Montanaro et al., 1995)

and gives rise to both α-DG and β-DG (Ibraghimov-Beskrovnaya *et al.*, 1992). The form of α-DG in the brain has an apparent molecular weight of 120 kDa and is presumably less glycosylated compared to the 156 kDa form in muscle (Gee *et al.*, 1993; Smalheiser and Kim, 1995). In the cerebellum α-DG is localized to Purkinje cell bodies and dendrites (Smalheiser and Kim, 1995) while in the retina α-DG is associated with the synapse-rich outer plexiform layer and with blood vessels (Montanaro *et al.*, 1995).

Given the presence of α-DG, DRP and laminins in cerebral blood vessels (Khurana *et al.*, 1992; Montanaro *et al.*, 1995; Jucker *et al.*, 1996a) and α-DG and dystrophin in a subpopulation of central nervous system (CNS) neurons (Gorecki *et al.*, 1994; Montanaro *et al.*, 1995), it is reasonable to speculate that α- and β-DG in the brain interact with dystrophin/DRP and/or laminin similarly as described in muscle. The present results localize DRP, α-DG, β-DG and laminin α2-chain to the glial–vascular interface, consistent with a molecular association of these molecules. The significance of this molecular association is supported by the observation that brain α-DG binds the α2-containing laminin-2 *in vitro*. In cerebellar Purkinje cells, the present results demonstrate α-DG associated with the cell surface and postsynaptic structures, suggesting a role for a dystrophin–dystroglycan complex in these neurons.

Materials and methods

Antibodies

The following antibodies were used: mouse monoclonal antibody (mAb) IIH6 specific to α-DG isolated from rabbit skeletal muscle membranes (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya *et al.*, 1992); mouse mAb to β-DG from human skeletal muscle (Novacastra Laboratories, Newcastle-upon-Tyne, UK); mouse mAb 2E8 to the laminin γ1 chain (Engvall *et al.*, 1990); and affinity-purified rabbit polyclonal antibody to the G-domain of the laminin α2-chain (Leivo and Engvall, 1988; Ehrig *et al.*, 1990). Specificity of α2 immunostaining in the brain with this antibody has been demonstrated previously (Jucker *et al.*, 1996b). Laminin antibodies were gifts of Dr E. Engvall (Ja Jolla Cancer Research Foundation, La Jolla, CA).

Tissue preparation for immunohistochemistry

Adult (12 months of age) F344 rats were used. All rats were overdosed with pentobarbital and perfused transcardially with PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, and postfixed overnight in the same fixative at 4°C. The cerebellum was then placed in 30% sucrose in PBS for 2 days at 4°C, and frozen in 2-methylbutane at –30°C. In some experiments cerebellar tissue from a similarly perfused rabbit and from an immersion-fixed pig brain were used.

Immunohistochemistry

Fixed brains were sectioned on a freezing–sliding microtome at 25 μm and collected in 0.05 M Tris buffer containing 1.5% NaCl, pH 7.4 (TB–NaCl). Sections were preincubated for 30 min in 1% H_2O_2. After extensive washing in TB–NaCl, sections were cut through the cerebellum and incubated in 0.3% Triton X-100 in TB–NaCl (10 min) followed by 5% horse serum (for monoclonal antibodies) or 5% goat serum (for polyclonal antibodies). Sections were reacted for 2 days at 4°C with primary antibodies diluted 1:500 to 1:3000 in TB–NaCl with 0.3% Triton and 2% serum. Tissue was washed with TB–NaCl for 3 × 10 min and incubated for 2 h in rat tissue-preabsorbed biotinylated horse anti-mouse IgG + IgM (1:100) or

biotinylated goat anti-rabbit IgG (1:400 in TB–NaCl; Vector Laboratories, Burlingame, CA). After washing, sections were incubated for 2 h in an avidin–biotin–peroxidase complex (ABC Elite Kit, Vector Laboratories). Tissue was washed again in TB–NaCl, and reacted with 0.05% 3'3-diaminobenzidine-HCl (Sigma), 0.06% $NiCl_2$ (Sigma), and 0.03% H_2O_2 in TB–NaCl. Subsequently, sections were washed, mounted on glass slides, air-dried, dehydrated, and coverslipped for light microscopy.

Control sections were treated in the same way but primary antibodies were omitted or replaced with mouse or rabbit IgG and IgM, all of which recognized unrelated antigens.

Immunoelectron microscopy

Rats were perfused transcardially with PBS followed by 4% paraform-aldehyde with various amounts of glutaraldehyde (0–1%) in 0.1 M phosphate buffer, as described above. Brains were removed, and postfixed for 2–12 h in the same fixative. Sections were cut with a vibratome (50 μm) and immunoreacted free-floating under the same conditions as described above but in most experiments without the use of Triton X-100. Sections were postfixed with 2% OsO_4 in PBS for 1 h, dehydrated in graded series of ethanol and propylene oxide, and flat-embedded between glass slides and coverslips (EMbedding Kit, Electron Microscopy Sciences, Fort Washington, PA). Semithin sections and thin sections were cut from the tissue surface on a ultramicrotome. Semithin sections (1 μm) were stained with toluidine blue for light microscopy. Thin sections were examined and photographed with a Hitachi 600 electron microscope.

Optimal fixation conditions for optimal ultrastructural preservation of the tissue did not reveal strong immunoreactivity for α-DG. Increasing postfixation intervals appeared to reduce blood vessel antigenicity for both α-DG and laminin (see also Jucker *et al.*, 1992). Moreover, increasing amounts of glutaraldehyde reduced the punctuated α-DG labelling in the molecular layer of the cerebellum (see Results). Labelling was always strong when Triton was used. The latter, however, is not compatible with a well-preserved ultrastructure.

Membrane protein isolation

Membrane proteins were isolated from the leg and back muscles of adult New Zealand White rabbits or the cerebellum of adult Sprague–Dawley rats by the protocol of Ohlendieck *et al.* (1991). Rabbit muscle was homogenized in a Polytron mixer (Brinkmann Instruments, Rexdale, Ontario, Canada) in 7.5 volumes of homogenization buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate mono-hydrate, 1 mM $MgCl_2$, 0.30 M sucrose, 0.5 mM EDTA, pH 7.0) in the presence of the following protease inhibitors: aprotinin (1 μM), leupeptin (1 μM), pepstatin A (1 μM), benzamidine (1 mM), iodoaceta-mide (1 mM) and phenylmethylsulphonyl fluoride (1 mM). The homogenate was centrifuged at 14 000 *g* at 4°C for 15 min. The supernatant was retained and the pellet was re-extracted in 75% of the original buffer volume. The supernatants were pooled and centrifuged at 30 000 *g* for 30 min at 4°C to pellet the heavy microsome fraction. The pellet was then resuspended in 0.6 M KCl, 0.30 M sucrose, 50 mM Tris–HCl, pH 7.4, with protease inhibitors, and held on ice for 30 min. Following incubation, this suspension was centrifuged at 142 000 *g* for 30 min at 4°C. The KCl-washed heavy microsomes (pellet) were solubilized on ice for 30 min in extraction buffer (1% digitonin, 50 mM Tris–HCl, pH 7.4) with protease inhibitors and centrifuged at 85 000 *g* for 30 min to pellet any insoluble material. The solubilized proteins were subsequently passaged over a succinylated wheatgerm agglutinin (sWGA)–agarose column overnight. The column was washed with five bed volumes

of the above protein extraction buffer with 0.1% digitonin and the bound fraction was eluted with 0.3 M N-acetyl-D-glucosamine in wash buffer.

Rat cerebellum was homogenized in a similar manner to that of the rabbit muscle with several modifications. Membrane pellets were obtained by centrifugation of cerebellar homogenates at 30 000 g for 30 min at 4°C. Pellets were not KCl-washed but rather directly resuspended in 1% digitonin extraction buffer with protease inhibitors and incubated on ice for 1 h. Insoluble material was removed by centrifugation at 85 000 g for 30 min at 4°C. Supernatant was aliquoted into Eppendorf tubes (1 ml per tube) and 100 µl of sWGA–agarose (Vector Laboratories) (beads 1:1 with digitonin extraction buffer) added. The resulting mixture was incubated at 4°C for 4 h with agitation. The sWGA–agarose beads were centrifuged, supernatants aspirated, and pelleted beads washed with the above extraction buffer. Following four additional washes the beads were eluted directly in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer.

Western blot analysis

Rabbit muscle and rat cerebellar sWGA-extracted fractions were electrophoretically separated on discontinuous SDS–PAGE gels with 4.0% stacking, 7.5% resolving phases (Laemmli, 1970). The separated proteins were subsequently electroblotted onto nitrocellulose membranes and blocked with 5% powdered skim milk in 10 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 0.1% Tween-20. The blot was probed for 30 min with mAb to α-DG or to β-DG diluted in blocking buffer. Blots were washed repeatedly for 1 h in blocking buffer minus the skim milk then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG + IgM antibody (Sigma). Excess secondary antibody was removed by repeated washes and bound antibody visualized by chemiluminescence (Dupont–NEN, Boston, MA).

Radiolabelled ligand blotting

α-Dystroglycan was purified from bovine brain by laminin-1 affinity chromatography as described previously (Gee et al., 1993). SDS–PAGE, electroblotting, radiolabelling of laminin-2 and radiolabelled ligand overlay assays were done essentially as described previously (Gee et al., 1993) with the following modifications. Laminin-2 from human placenta (Gibco/BRL) was labelled with ^{125}I and diluted to a final concentration of 100 ng/ml. Competition of [^{125}I]laminin-2 binding to brain α-DG was accomplished with excess unlabelled laminin-1 instead of laminin-2.

Results

Light microscopic immunolocalization of α- and β-DG in rat cerebellum

All layers and white matter tracts of the cerebellum revealed distinct labelling of blood vessels with both α-DG- and β-DG-specific antibodies (Fig. 1A, C). In addition, labelling for α-DG was observed throughout the entire molecular layer (Fig. 1A). Under high-power light microscopy, α-DG immunolabelling in the molecular layer appeared punctate with additional labelling of fibres (Fig. 1A, B). In rabbit and pig cerebellum an even more distinct punctate and fibre-like staining of the molecular layer was found (not shown). Immunoreactivity for α-DG was also associated with the cell surface and proximal dendrites of Purkinje cells (Fig. 1B). The cell surface-associated α-DG immunoreactivity appeared interrupted (Fig. 1B). Rarely, some α-DG immunoreactivty was observed in the cytoplasm of the Purkinje cells. No convincing β-DG immunoreactivity was observed in the molecular layer and Purkinje cell layer except in blood vessels (Fig. 1C).

Ultrastructural localization of α- and β-DG and of α2-chain-containing laminin in blood vessels

Immunoelectron microscopy of capillaries in the rat cerebellum revealed α-DG associated with the entire basement membrane at the glial–vascular interface (parenchymal basement membrane) (Fig. 2A, B). No immunolabelling was associated with the basement membrane between endothelial cells and pericytes (Fig. 2A, B), suggesting that α-DG is not associated with the endothelial basement membrane unless it makes contact with the endfeet of the perivascular astrocytes (fused endothelial–parenchymal basement membrane; Peters et al., 1991). Similarly, in arterioles the basement membrane between endothelial and smooth muscle cells and between smooth muscle cell and smooth muscle cell was always free of immunoreactivity (not shown).

β-Dystroglycan at the ultrastructural level was localized to astrocytic endfeet, which often completely surround the parenchymal basement membrane of blood vessels (Fig. 2C, D). No labelling of endothelial cells, pericytes, endothelial basement membrane or parenchymal basement membrane was observed.

We have shown previously in the mouse that the laminin α2-chain is a component of the cerebral parenchymal vascular basement membrane (Jucker et al., 1996b). To assess whether α-DG might be a receptor for α2-chain-containing laminins in the parenchymal basement membrane, we re-examined the ultrastructural localization of the laminin α2-chain in sections adjacent to the α-DG-immunostained sections in the rat cerebellum. Results revealed laminin α2-chain immunoreactivity confined to the parenchymal basement membrane (Fig. 2E, F). No laminin α2-chain immunoreactivity was associated with the endothelial basement membrane between endothelial cells and pericytes (Fig. 2E, F), demonstrating that α2-chain-containing laminins and α-DG are confined to and colocalized at the parenchymal vascular basement membrane. In contrast, when mAb 2E8 against the γ1 chain of laminin was used, labelling of both parenchymal and endothelial basement membranes was observed (not shown, but see Jucker et al., 1996a).

Ultrastructural localization of α-DG in the molecular layer and Purkinje cell layer of rat cerebellum

Electron microscopic analysis of Purkinje cells revealed α-DG immunoreactivity associated with the plasma membrane of Purkinje cell bodies (Fig. 3A). The immunoreactivity over the cell surface appeared patchy. Since fixation conditions for the ultrastructural analysis were not optimal (see Materials and methods), it was often difficult to recognize the labelled structures in detail. Sometimes the reaction product also appeared associated with neighbouring structures—often astrocytic processes—and on rare occasions we also observed some cytoplasmic labelling of Purkinje cells. In the molecular layer the reaction product was localized to postsynaptic structures (dendritic spines) with some labelling of dendritic processes (Fig. 3B, C). Only a subpopulation of dendritic spines appeared labelled, and the reaction product was clearly more intense towards the postsynaptic density (Fig. 3C).

Monoclonal antibodies recognize α-DG and β-DG in rat cerebellum

Specificity of immunostaining with mAbs to α-DG and to β-DG was assessed by immunoblot analysis of the adult rat cerebellum (Fig. 4). In initial immunoblot experiments with whole cerebellar homogenates,
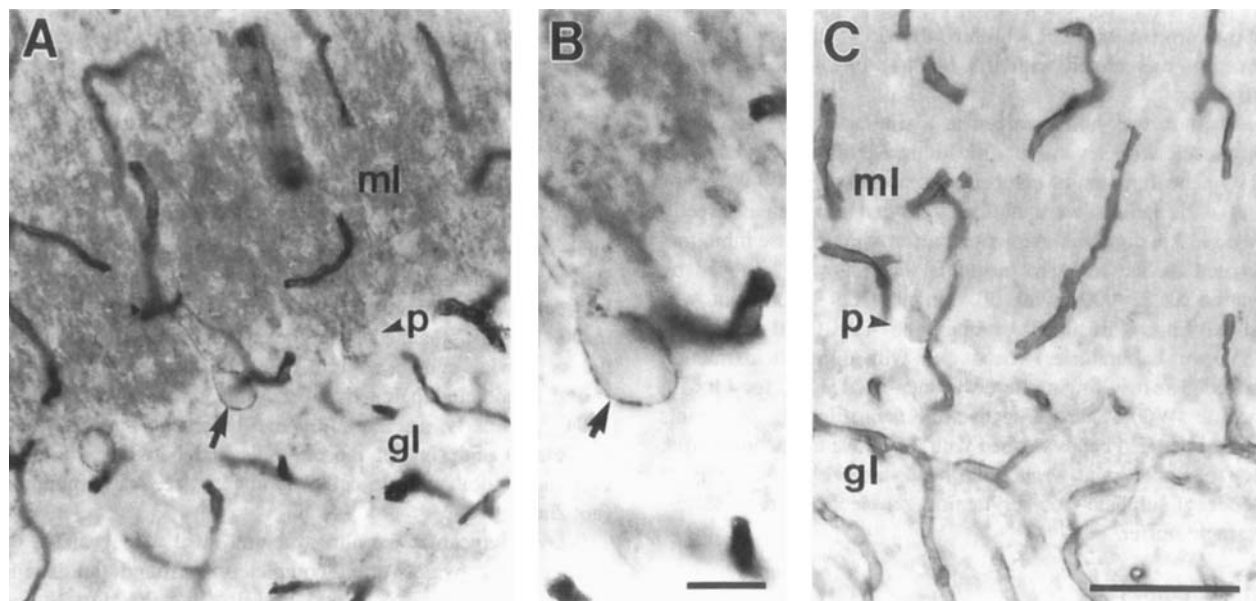
FIG. 1. Localization of α-DG (A, B) and β-DG (C) in adult rat cerebellum. (A) Immunolabelling for α-DG in coronal sections reveals labelling of blood vessels in the molecular layer (ml), Purkinje cell layer (p), and granule cell layer (gl). Additional punctate α-DG labelling is present in the molecular layer. Distinct labelling was also associated with the Purkinje cell surface (arrow) and proximal dendritic processes. (B) Area indicated by an arrow in A is shown at higher magnification. Note that immunoreactivity associated with the cell surface appears to be interrupted or at least not homogeneously distributed (arrow). Note also the immunolabelling associated with the proximal dendritic process. (C) Immunolabelling for β-DG reveals strong labelling of blood vessels throughout all layers of the cerebellum. A and C have the same magnification. Scale bars, 25 μm (B) and 100 μm (C).

we did not observe specific bands with mAb IIH6 to α-DG (not shown). However, after enriching α-DG by using a membrane protein fraction of the cerebellum, mAb IIH6 to α-DG revealed a distinct single band at ~120 kDa (Fig. 4A, lane 2) which is in agreement with the reported 120 kDa molecular weight for α-DG in the brain (Ibraghimov-Beskrovnaya et al., 1992; Gee et al., 1993; Smalheiser and Kim, 1995). For comparison, the same antibody revealed the expected and previously reported 156 kDa band in rabbit muscle (Fig. 4A, lane 1; Ibraghimov-Beskrovnaya et al., 1992). Under the same conditions mAb to β-DG revealed a 43 kDa band in the brain which was also found in muscle (Fig. 4B).

Brain α-DG binds laminin-2

It has been demonstrated that laminin-1 binds brain α-DG (Gee et al., 1993) and that laminin-2 binds to α-DG from peripheral nerve (which is similar in molecular weight to brain α-DG; Yamada et al., 1994). However, binding of the α2-chain-containing laminin-2 to brain α-DG has not yet been demonstrated. Here we show that in blots of purified α-DG from brain probed with radiolabelled laminin-2, a band essentially identical to the 120 kDa band of brain α-DG was found to bind specifically to laminin-2 (Fig. 5).

Discussion

Results of the present study suggest that dystroglycan in the cerebellum is associated with both blood vessels and neuronal structures. In blood vessels α-DG is localized to the parenchymal vascular basement membrane while β-DG is present in perivascular astrocytic endfeet. In the Purkinje cell layer and molecular layer of the cerebellum, α-DG is associated with the surface of Purkinje cell bodies and dendrites, and is concentrated in dendritic spines.

Previous studies have localized DRP in the brain to the inner plasma face of endfeet of perivascular astrocytes (Khurana et al., 1992). In the present study we show that β-DG is also localized to perivascular astrocytic endfeet. Moreover, we demonstrate that α-DG is associated with the laminin α2-chain-rich parenchymal vascular basement membrane upon which the perivascular astrocytic endfeet rest. Given the dystrophin/DRP–dystroglycan–laminin α2 linkage of the cell cytoskeleton to the overlying basement membrane in skeletal muscle (Ahn and Kunkel, 1993; Campbell, 1995), the localization of DRP, α-DG, β-DG and the laminin α2-chain at the glial–vascular interface suggests a similar linkage between astrocytic endfeet and parenchymal vascular basement membrane in the cerebellum. A broad immunohistochemical survey throughout the brain revealed that α-DG and β-DG were associated with blood vessels throughout the brain (not shown), suggesting that the dystroglycan-mediated linkage between astrocytic endfeet and vascular basement membrane is not limited to the cerebellum.

α-Dystroglycan and β-DG are derived from a common precursor protein and copurify in the brain (Ibraghimov-Beskrovnaya et al., 1992; Smalheiser and Kim, 1995). While α-DG is an extracellular membrane-associated glycoprotein, β-DG is a transmembrane glyco-protein (Ibraghimov-Beskrovnaya et al., 1992). In muscle, α- and β-DG are associated with several other glycoproteins. However, it has been demonstrated that the tightly complexed α- and β-DG can mediate alone the linkage between DRP and laminin (Yoshida et al., 1994). A dystroglycan-mediated linkage between perivascular astrocytes and the laminin α2-chain-rich parenchymal vascular basement membrane is consistent with conclusions from a recent study which reported immunoprecipitation of a DRP–laminin complex from neonatal astrocytes with antibodies either to laminin or to DRP (Khurana et al., 1995).

Recent evidence suggests the presence of multiple laminin variants in cerebral vascular basement membranes (Jucker et al., 1996a).
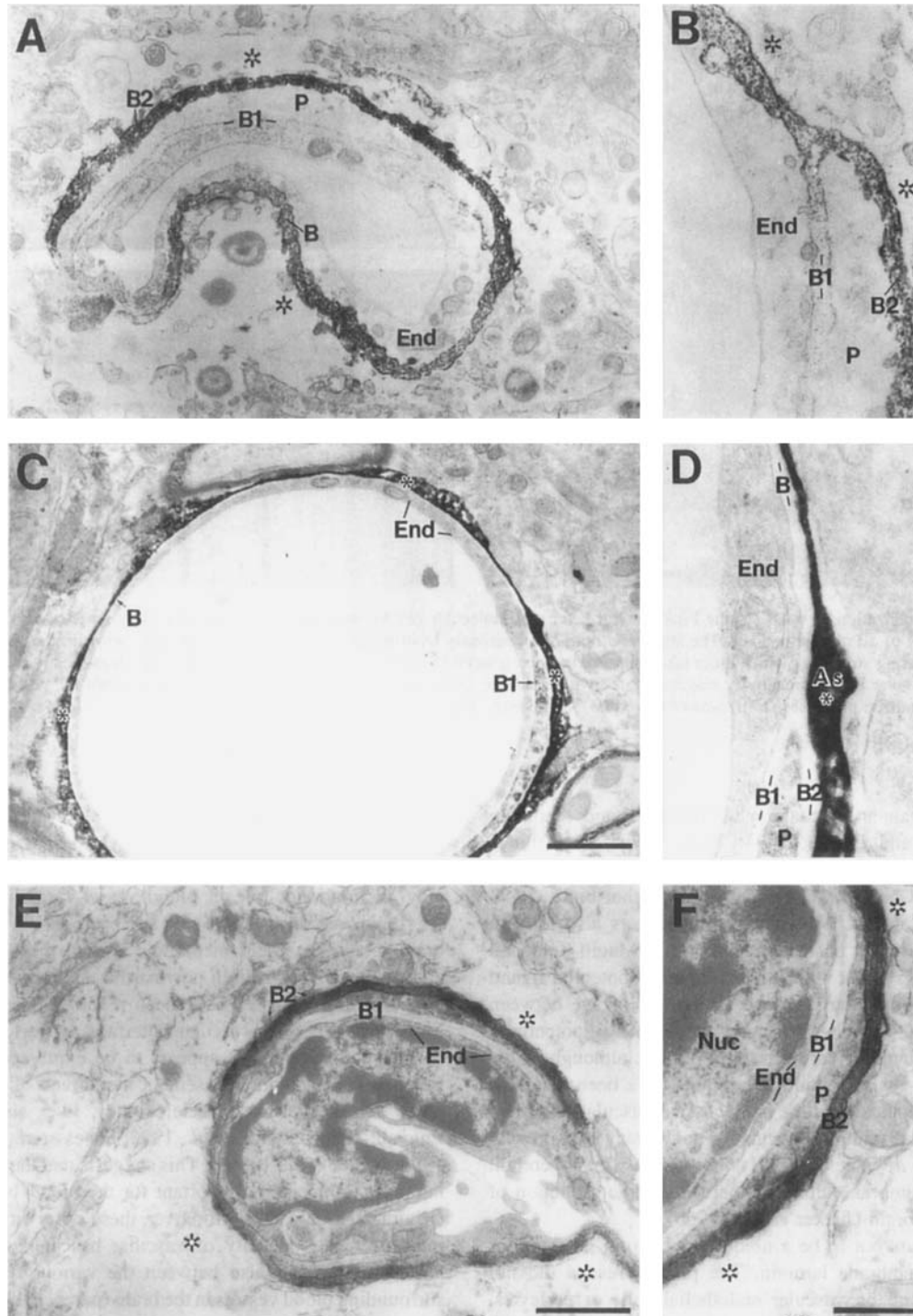
FIG. 2. Ultrastructural localization of α-DG (A, B), β-DG (C, D) and laminin α2 (E, F) in blood vessels of rat cerebellum. Brain capillary endothelial cells (End) are surrounded by basement membranes. If pericytes are absent only one basement membrane is observed (B; fused endothelial–parenchymal basement membrane). Two basement membranes (B1, endothelial basement membrane; B2, parenchymal basement membrane) are, however, always observed where pericytes (P) occur (Peters *et al.*, 1991). Astrocytic endfeet (asterisks) usually cover the entire abluminal parenchymal basement membrane and form an almost complete limiting membrane. (A, B) Immunoreactivity for α-DG is confined to the fused parenchymal–endothelial basement membrane (B) and to the parenchymal basement membrane proper (B2) while the endothelial basement membrane (B1) appears unlabelled. Astrocytic endfeet are also unlabelled and are poorly preserved due to the suboptimal tissue fixation that was necessary to get reliable α-DG immunolabelling (see Materials and methods). (C, D) Immunoreactivity for β-DG is confined to the astrocytic endfeet surrounding the parenchymal basement membrane. Astrocytic endfeet appear much narrower compared with panels A, B and E, F, since the ultrastructure of the tissue is better preserved. Endothelial (B1) and parenchymal (B2) basement membranes as well as endothelial cells and pericytes are unlabelled. (E, F) Immunoreactivity to the laminin α2-chain is associated with the parenchymal basement membrane (B2) but not with the endothelial basement membrane (B1), very similar to α-DG. Shown is a collapsed capillary with a prominent endothelial cell nucleus (Nuc). Scale bars, 1 μm (C, E) and 0.5 μm (F). A, E and B, D, F have the same magnifications.
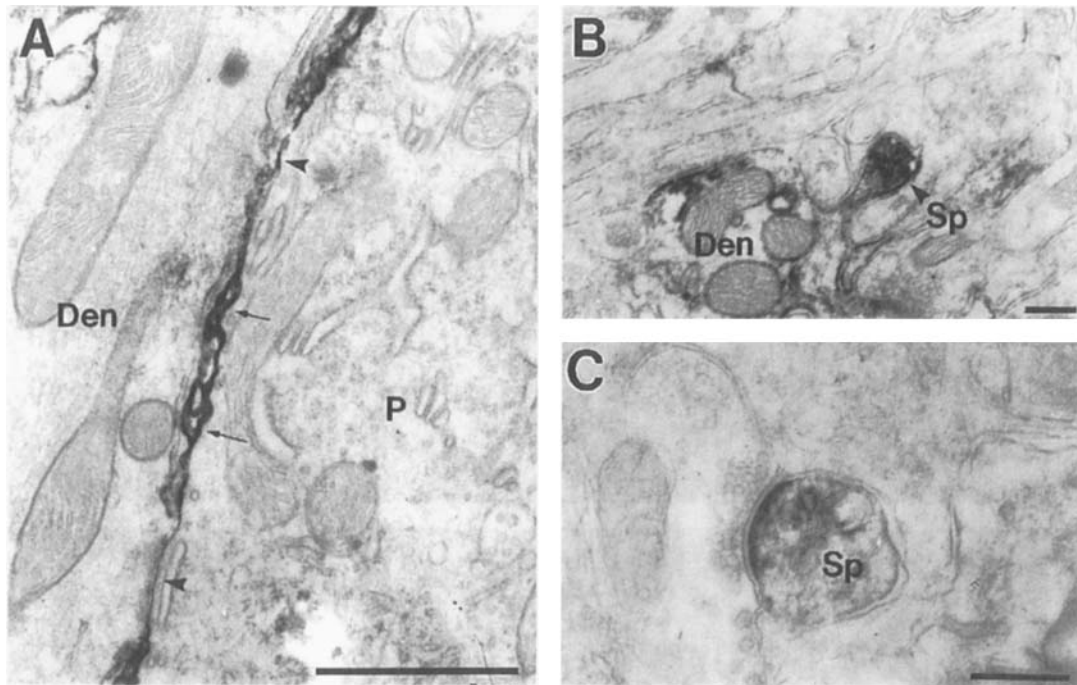
FIG. 3. Ultrastructural localization of α-DG in the Purkinje cell layer and molecular cell layer of rat cerebellum. (A) Immunoreactivity for α-DG is associated with the surface (arrows) of a Purkinje cell (P). The surface is not homogeneously labelled and labelling appears patchy (arrowheads). Often labelling was also associated with neighbouring structures which quite often appeared to be astrocytic processes. The Purkinje cell cytoplasm was mostly free of immunoreactivity. (B, C) In the molecular layer immunoreactivity was associated with dendritic spines (Sp), with more intense labelling towards the postsynaptic density (C). In addition, labelling of dendritic processes (Den) was observed (B). Scale bars, 1 μm (A) and 0.25 μm (B, C).

Laminin-1 and -3 contain an α1 chain, while laminin-2 and -4 contain an α2-chain (Timpl and Brown, 1994). Since brain α-DG binds laminin-1 and laminin-2 through the C-terminal α1 and α2 G-domains respectively (Fig. 5; Gee et al., 1993), it is likely that brain α-DG also binds laminin-3 and -4. Moreover, agrin appears also to be a component of the vascular basement membrane (Magill-Solc and McMahan, 1988; Rupp et al., 1991) and it is also a potential ligand for α-DG at the glial–vascular interface. Thus, the linkage between astrocytic endfeet and vascular basement membrane is potentially mediated by several laminin variants and by agrin, although at the present time only α2-chain-containing laminins have been localized unequivocally and specifically to the parenchymal vascular basement membrane. The view of multiple ligands for α-DG may explain why α2 mutant dystrophic dy mice show no abnormalities in the cerebral vascular basement membrane although there is a large reduction of α2 expression in the brain (Jucker et al., 1996b).

Dystroglycan appears not to be a ubiquitous laminin receptor for cerebral basement membrane laminin. The present results did not reveal β-DG expression on vascular endothelial cells or pericytes, nor did we observe α-DG associated with the endothelial basement membrane proper, i.e. the basement membrane observed between endothelial cells and pericytes. Similarly, α- and β-DG were not observed on vascular smooth muscle cells and were not associated with smooth muscle basement membrane, respectively. Other laminin receptors, notably integrin receptors, might mediate the linkage between basement membrane laminin and endothelial and smooth muscle cells (Grooms et al., 1993; Timpl and Brown, 1994; Khurana et al., 1995).

Dystroglycan-mediated linkage between perivascular astrocytes and vascular basement membrane is probably important for the integrity of the vascular–glial interface. In the developing kidney, the expression

of α-DG on epithelial cells was suggested to act as a nucleation site for basement membrane assembly (Durbeej et al., 1995). Similarly, in the developing and injured brain, dystroglycan on astrocytes might play an important role in blood vessel formation and stabilization (cf. Lawrence et al., 1984). It has been shown that in the lesioned brain a new basement membrane around the scar tissue is formed and polymerizing laminin-positive basement membrane components on astrocytic processes have been reported (Bernstein et al., 1985). Moreover, the glial–vascular interface is part of the blood–brain barrier, and laminin α2 appears to be expressed specifically in the vascular (parenchymal) basement membrane of the brain but not of other organs such as muscle, kidney, liver and heart (Leivo and Engvall, 1988; Engvall et al., 1990; Sanes et al., 1990; Wewer et al., 1992; Jucker et al., 1996b). This suggests that the DRP–dystroglycan–laminin α2 linkage is important for the blood–brain barrier function (Khurana et al., 1992). Moreover, these observations demonstrate the molecular heterogeneity of vascular basement membrane not only among tissues but also between the various basement membranes surrounding blood vessels in the brain (parenchymal versus endothelial and smooth muscle basement membrane).

Beside the glial–vascular localization, dystroglycan in the brain was reported to be expressed in neurons of the retina, olfactory bulb, thalamus, hippocampus, dentate gyrus and cerebellum (Gorecki et al., 1994; Montanaro et al., 1995). In the cerebellum dystroglycan mRNA is restricted to Purkinje cells (Gorecki et al., 1994) and the present results show that α-DG is associated with the Purkinje cell surface, and with dendrites and dendritic spines. In Purkinje cell dendrites and dendritic spines the reaction product appeared primarily intracellular although an additional cell surface localization is possible and was apparent at the light microscopic level. Smalheiser and Kim (1995) have also reported labelling of Purkinje cell bodies and fibres at the
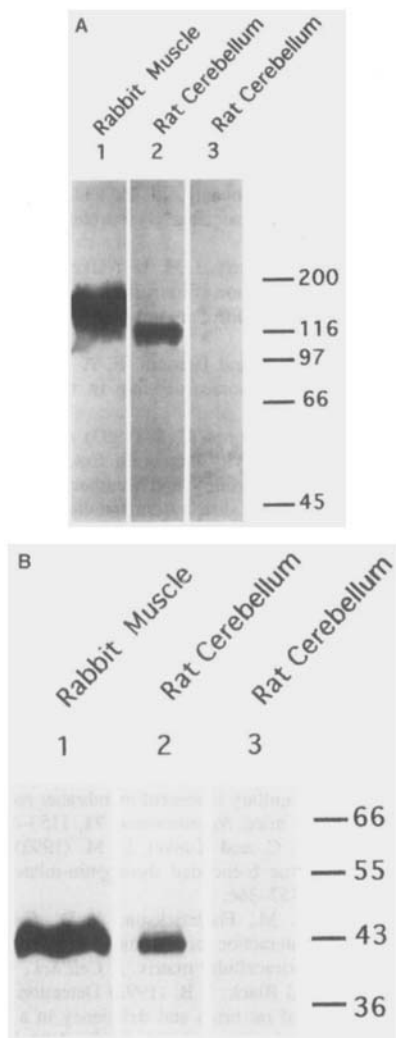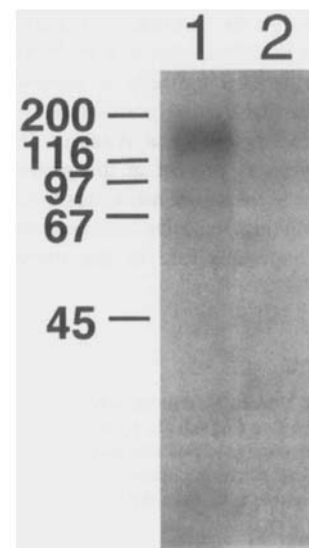
FIG. 5. Laminin-2 binding to purified brain α-DG. Purified α-DG from brain was electrophoresed, transferred to nitrocellulose, and probed with ^{125}I-labelled laminin-2 in the absence (lane 1) or presence (lanc 2) of a 1000-fold excess of unlabelled laminin-1. Molecular weight standards are indicated at the left (in kDa).

FIG. 4. Western blot analysis of rat cerebellum and rabbit muscle. (A) Nitrocellulose transfers of sWGA-isolated rat and rabbit membrane proteins that had been electrophoretically separated under reducing conditions were probed with monoclonal antibody (mAb) IIH6. This mAb interacts with a glycosylated region of α-DG (Ervasti and Campbell, 1993) and recognizes a band at ~150–160 kDa in rabbit muscle (lane 1) and ~120 kDa in the rat cerebellum (lane 2). Lane 3 was probed with secondary antibody alone. (B) Western blot analysis of rat cerebellum and rabbit muscle with mAb to β-DG under the same conditions revealed a 43 kDa band in both rat cerebellum (lane 2) and rabbit muscle (lane 1). Lane 3 was probed with secondary antibody alone. Molecular mass markers (in kDa) are shown to the right.

light microscopic level with a peptide antibody to α-DG. The latter antibody, however, revealed more prominent intracellular labelling of the Purkinje cell bodies and did not reveal blood vessel labelling. This apparent discrepancy is probably the result of differences in tissue fixation. For example, we have found that increased fixation reduces labelling for α-DG at the glial–vascular interface (see Materials and methods) and that fixation tends to increase non-specific intracellular labelling of Purkinje cell bodies, the latter being also appreciated by Smalheiser and Kim (1995).

Although we have not convincingly observed neuronal staining with the monoclonal antibody to β-DG used in the present study, others have reported the presence of β-DG in synaptic membrane fractions of brain (Mummery et al., 1996). Since the dystroglycan transcript is expressed in Purkinje neurons and gives rise to both α-DG and β-DG (Ibraghimov-Beskrovnaya et al., 1992; Gorecki et al.,

1994). It is possible that the lack of neuronal β-DG staining in the present study is due to post-translational protein modification, epitope masking, or other more technical reasons rather than reflecting the absence of β-DG in Purkinje cells.

Purkinje cells also express dystrophin and the protein has been localized to the inside of the plasma membrane, where it occurs in punctate aggregates (Lidov et al., 1993; Gorecki et al., 1994). Dystrophin is also enriched in postsynaptic sites (Lidov et al., 1990; Kim et al., 1992). These observations and the fact that dystrophin and dystroglycan transcripts are co-expressed in cerebellar Purkinje cells (Gorecki et al., 1994) might indicate the presence of a dystrophin–dystroglycan-like complex in these neurons. It is also possible that dystroglycan interacts with truncated dystrophin and DRP isoforms which have been described in the brain (Lederfein et al., 1992; Ahn and Kunkel, 1993; Schofield et al., 1994; Blake et al., 1995). Since they all lack the putative actin-binding domain, it is possible that they mediate novel or different functions.

Dystroglycan associated with Purkinje cells probably does not interact with extracellular laminin in the normal adult brain since there is no evidence for laminins in the perineuronal space (Sanes 1989; Jucker et al., 1996a). In contrast, during brain development extracellular deposits of laminin are transiently observed and laminin-1 and -2 have been implicated in cell adhesion, cell migration and neurite outgrowth (Sanes, 1989; Zhou, 1990; Calof and Lander 1991; Letourneau et al., 1994). α-Dystroglycan in adult brain, however, might bind and interact with other laminin G-domain-containing proteins in the CNS, such as agrin (Rupp et al., 1991; O'Connor et al., 1994) and the synaptic membrane proteins neurexins (Ushkaryov et al., 1992; Ushkaryov and Südhof, 1993). While binding of α-DG to neurexins has not yet been examined, α-DG interacts with agrin in muscle, and this interaction appears to be important for myosynapse organization (Gee et al., 1994; Campanelli et al., 1994; Bowe et al., 1994). However, it is also conceivable that α-DG functions intracellularly and interacts with intraneuronal laminin-like molecules (Hagg et al., 1989) or the laminin α2-chain-like molecules recently observed in dendrites and dendritic spines (Tian et al., 1994).

In conclusion, although the function of dystroglycan in neurons is not yet clear, the fact that dystroglycan and dystrophin appear to be colocalized in cerebellar Purkinje cells, in particular at postsynaptic sites, supports the idea that dystroglycan interacts with dystrophin in a subpopulation of CNS neurons and synapses. Besides its neuronal expression, dystroglycan is present at the glial–vascular interface, where it links DRP in perivascular astrocytes to α2-chain-containing laminin in the parenchymal vascular basement membrane, and thus probably plays an important role in the integrity of the glial–vascular interface.

Acknowledgements

Abbreviations

α-DG	α-dystroglycan
β-DG	β-dystroglycan
CNS	central nervous system
DRP	dystrophin-related protein
mAb	monoclonal antibody
SDS–PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
sWGA	succinylated wheatgerm agglutinin

References

Ahn, A. H. and Kunkel, L. M. (1993) The structure and functional diversity of dystrophin. *Nature Genet.*, 3, 283–291.

Bernstein, J. J., Getz, R., Jeffereson, M. and Keleman, M. (1985) Astrocytes secrete basal lamina after hemisections of rat spinal cord. *Brain Res.*, 327, 135–141.

Billard, C., Gillet, P., Signoret, J. L., Uicaut, E., Bertrand, P., Fardeau, M., Barthez-Carpentier, M. A. and Santini, J. J. (1992) Cognitive function in Duchenne muscular dystrophy: a reappraisal and comparison with spinal muscular atrophy. *Neuromusc. Disord.*, 2, 371–378.

Blake, D. J., Schofield, J. N., Zuellig, R. A., Gorecki, D. C., Phelps, S. R., Barnard, E. A., Edwards, Y. H. and Davies, K. E. (1995) G-utrophin, the autosomal homologue of dystrophin Dp116, is expressed in sensory ganglia and brain. *Proc. Natl Acad. Sci. USA*, 92, 3697–3701.

Bowe, M. A., Deyst, K. A., Leszyk, J. D. and Fallon, J. R. (1994) Identification and purification of an agrin receptor from *Torpedo* postsynaptic membranes: a heteromeric complex related to the dystroglycans. *Neuron*, 12, 1173–1180.

Calof, A. L. and Lander, A. D. (1991) Relationship between neuronal migration and cell–substratum adhesion: laminin and merosin promote olfactory neuronal migration but are anti-adhesive. *J. Cell Biol.*, 115, 779–794.

Campanelli, J. T., Roberds, S. L., Campbell, K. P. and Scheller, R. H. (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell*, 77, 663–674.

Campbell, K. P. (1995) Three muscular dystrophies: loss of cytoskeleton–extracellular matrix linkage. *Cell*, 80, 675–679.

Douville, P. J., Harvey, W. J. and Carbonetto, S. (1988) Isolation and partial characterization of high affinity laminin receptors in neural cells. *J. Biol. Chem.*, 263, 14964–14969.

Durbeej, M., Larsson, E., Ibraghimov-Beskrovnaya, O., Roberds, S., Campbell, K. P. and Ekblom, P. (1995) Non-muscle α-dystroglycan is involved in epithelial development. *J. Cell Biol.*, 130, 79–91.

Ehrig, K., Leivo, I., Agraves, W. S., Ruoslathi, E. and Engvall, E. (1990) Merosin, a tissue-specific basement membrane protein, is a laminin-like protein. *Proc. Natl Acad. Sci. USA*, 87, 3264–3268.

Engvall, E., Earwicker, D., Haaparanta, T., Ruoslathi, E. and Sanes, J. R. (1990) Distribution and isolation of four laminin variants; tissue restricted distribution of heterotrimers assembled from five different subunits. *Cell Reg.*, 1, 731–740.

Ervasti, J. M. and Campbell, K. P. (1991) Membrane organization of the dystrophin–glycoprotein complex. *Cell*, 66, 1121–1131.

Ervasti, J. M. and Campbell, K. P. (1993) A role for the dystrophin–glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.*, 122, 809–823.

Gee, S. H., Blacher, R. W., Douville, P. J., Provost, P. R., Yurchenco, P. D. and Carbonetto, S. (1993) Laminin-binding protein 120 from brain is closely related to the dystrophin-associated glycoprotein, dystroglycan, and binds with high affinity to the major heparin binding domain of laminin. *J. Biol. Chem.*, 268, 14972–14980.

Gee, S. H., Montanaro, F., Lindenbaum, M. H. and Carbonetto, S. (1994) Dystroglycan-α, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell*, 77, 675–686.

Gorecki, D. C., Monaco, A. P., Derry, J. M. J., Walker, A. P., Barnard, E. A. and Barnard, P. J. (1992) Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters. *Hum. Mol. Genet.*, 1, 505–510.

Gorecki, D. C., Derry, J. M. J. and Barnard, E. A . (1994) Dystroglycan: brain localization and chromosome mapping in the mouse. *Hum. Mol. Genet.*, 3, 1589–1597.

Grooms, S. Y., Terracio, L. and Jones, L. S. (1993) Anatomical localization of β1 integrin-like immunoreactivity in rat brain. *Exp. Neurol.*, 122, 253–259.

Hagg, T., Muir, D., Engvall, E., Varon, S. and Manthorpe, M. (1989) Laminin-like antigen in rat CNS neurons: distribution and changes upon brain injury and nerve growth factor treatment. *Neuron*, 3, 721–732.

Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Semett, S. W. and Campbell, K. P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature*, 355, 696–702.

Jucker, M., Bialobok, P., Hagg, T. and Ingram, D. K. (1992) Laminin immunohistochemistry in brain is dependent on the method of tissue fixation. *Brain. Res.*, 586, 166–170.

Jucker, M., Tian, M. and Ingram, D. K. (1996a) Laminins in the adult and aged brain. *Mol. Chem. Neuropathol.*, 28, 209–218.

Jucker, M., Tian, M., Norton, D. D., Sherman, C. and Kusiak, J. (1996b) Laminin α2 in the CNS capillary basement membrane: reduced expression in brains of dystrophic *dy* mice. *Neuroscience*, 71, 1153–1161.

Khurana, T. S., Watkins, S. C. and Kunkel, L. M. (1992) The subcellular distribution of chromosome 6-encoded dystrophin-related protein in the brain. *J. Cell Biol.*, 119, 357–366.

Khurana, T. S., Kunkel, L. M., Frederickson, A. D., Carbonetto, S. and Watkins, S. C. (1995) Interaction of chromosome 6-encoded dystrophin related protein with the extracellular matrix. *J. Cell Sci.*, 108, 173–185.

Kim, T., Wu, K., Xu, J. and Black, I. B. (1992) Detection of dystrophin in the postsynaptic density of rat brain and deficiency in a mouse model of Duchenne muscular dystrophy. *Proc. Natl Acad. Sci. USA*, 89, 11642–11644.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, 227, 680–685.

Lawrence, J. M., Huang, S. K. and Raisman, G. (1984) Vascular and astrocytic reactions during establishment of hippocampal transplants in adult host brain. *Neuroscience*, 12, 745–760.

Lederfein, D., Levy, Z., Augier, N., Mornet, D., Morris, G., Fuchs, O., Yaffe, D. and Nudel, U. (1992) A 71-kilodalton protein is the major product of the Duchenne muscular dystrophy gene in brain and other nonmuscle tissues. *Proc. Natl Acad. Sci. USA*, 89, 5346–5360.

Leivo, I. and Engvall, E. (1988) Merosin, a protein specific for basement membranes of Schwann cells, striated muscle, and trophoblast, is expressed late in nerve and muscle development. *Proc. Natl Acad. Sci. USA*, 85, 1544–1548.

Letourneau, P. C., Condic, M. L. and Snow, D. M. (1994) Interactions of neurons with the extracellular matrix. *J. Neurosci.*, 14, 915–928.

Lidov, H. G. W., Byers, T. J., Watkins, S. C. and Kunkel, L. M. (1990) Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. *Nature*, 348, 725–728.

Lidov, H. G. W., Byers, T. J. and Kunkel, L. M. (1993) The distribution of dystrophin in the murine central nervous system: an immunocytochemical study. *Neuroscience*, 54, 167–187.

Magill-Solc, C. and McMahan, U. J. (1988) Motor neurons contain agrin-like molecules. *J. Cell Biol.*, 107, 1825–1833.

Matsumara, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D. and Campbell, K. P. (1992) Association of dystrophin related protein with dystrophin-associated proteins in *mdx* mouse muscle. *Nature*, 360, 588–591.

Montanaro, F., Carbonetto, S., Campbell, K. P. and Lindenbaum, M. (1995) Dystroglycan expression in the wild type and *mdx* mouse neural retina: synaptic colocalization with dystrophin, dystrophin-related protein but not laminin. *J. Neurosci. Res.*, 42, 528–538.

Mummery, R., Sessay, A., Lai, F. A. and Beesley, P. W. (1996) β-Dystroglycan: subcellular localisation in rat brain and detection of a novel immunologically

related, postsynaptic density-enriched protein. *J. Neurochem.*, **66**, 2455–2459.

O'Connor, L. T., Lauterborn, J. C., Gall, C. M. and Smith, M. A. (1994) Localization and alternative splicing of agrin mRNA in adult rat brain: transcripts encoding isoforms that aggregate acetylcholine receptors are not restricted to cholinergic regions. *J. Neurosci.*, **14**, 1141–1152.

Ohlendieck, K., Ervasti, J. M., Snook, S. D. and Campbell, K. P. (1991) Dystrophin–glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. *J. Cell Biol.*, **112**, 135–148.

Peters, A., Palay, S. L. and Webster, H. deF. (1991) *The Fine Structure of the Nervous System. Neurons and Their Supporting Cells,* 3rd edn. Oxford University Press, New York.

Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M. and Scheller, R. H. (1991) Structure and expression of a rat agrin. *Neuron*, **6**, 811–823.

Sanes, J. R. (1989) Extracellular matrix molecules that influence neural development. *Annu. Rev. Neurosci.*, **12**, 491–516.

Sanes, J. R., Engvall, E., Butkowski, R. and Hunter, D. D. (1990) Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. *J. Cell Biol.*, **111**, 1685–1699.

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 Duchenne muscular dystrophy locus: expression during mouse embryogenesis and in cultured cell lines. *Hum. Mol. Gen.*, **3**, 1309–1316.

Smalheiser, N. R. and Kim, E. (1995) Purification of cranin, a laminin binding membrane protein. *J. Biol. Chem.*, **270**, 15425–15433.

Smalheiser, N. R. and Schwartz, N. B. (1987) Cranin: A laminin-binding protein of cell membranes. *Proc. Natl Acad. Sci. USA*, **84**, 6457–6461.

Tian, M., Hagg, T., Engvall, E., Hall, H. and Jucker, M. (1994) Merosin (M-chain)-like antigens in dendritic spines of the normal, developing, and regenerating CNS. *Soc. Neurosci. Abstr.*, **20**, 865.

Timpl, R. and Brown, J. C. (1994) The laminins. *Matrix Biol.*, **14**, 275–281.

Ushkaryov, Y. A. and Südhof, T. C. (1993) Neurexins IIIα: extensive alternative splicing generates membrane-bound and soluble forms. *Proc. Natl Acad. Sci. USA*, **90**, 6410–6414.

Ushkaryov, Y. A., Petrenko, A. G., Geppert, M. and Südhof, T. C. (1992) Neurexins: synaptic cell surface proteins related to the α-latrotoxin receptor and laminin. *Science*, **257**, 50–56.

Yamada, H., Shimizu, T., Tanaka, T., Campbell, K. P. and Matsumara, K. (1994) Dystroglycan is a binding protein of laminin and merosin in peripheral nerve. *FEBS Lett.*, **352**, 49–53.

Yoshida, M., Suzuki, A., Yamamoto, H., Noguchi, S., Mizuno, Y. and Ozawa, E. (1994) Dissociation of the complex of dystrophin and its associated proteins into several unique groups by *n*-octyl β-D-glucosidase. *Eur. J. Biochem.*, **222**, 1055–1961.

Wewer, U. M., Engvall, E., Paulsson, M., Yamada, M. and Albrechtsen, R. (1992) Laminin A, B1, B2, S and M subunits in the postnatal rat liver development and after partial hepatectomy. *Lab. Invest.*, **66**, 378–389.

Zhou, F. C. (1990) Four patterns of laminin-immunoreactive structures in developing rat brain. *Dev. Brain Res.*, **55**, 191–201.