

Different Dystrophin-like Complexes Are Expressed in Neurons and Glia

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Abstract. Duchenne muscular dystrophy is a fatal muscle disease that is often associated with cognitive impairment. Accordingly, dystrophin is found at the muscle sarcolemma and at postsynaptic sites in neurons. In muscle, dystrophin forms part of a membrane-spanning complex, the dystrophin-associated protein complex (DPC). Whereas the composition of the DPC in muscle is well documented, the existence of a similar complex in brain remains largely unknown. To determine the composition of DPC-like complexes in brain, we have examined the molecular associations and distribution of the dystrobrevins, a widely expressed family of dystrophin-associated proteins, some of which are components of the muscle DPC. β -Dystrobrevin is found in neurons and is highly enriched in postsynaptic densities

(PSDs). Furthermore, β -dystrobrevin forms a specific complex with dystrophin and syntrophin. By contrast, α -dystrobrevin-1 is found in perivascular astrocytes and Bergmann glia, and is not PSD-enriched. α -Dystrobrevin-1 is associated with Dp71, utrophin, and syntrophin. In the brains of mice that lack dystrophin and Dp71, the dystrobrevin-syntrophin complexes are still formed, whereas in dystrophin-deficient muscle, the assembly of the DPC is disrupted. Thus, despite the similarity in primary sequence, α - and β -dystrobrevin are differentially distributed in the brain where they form separate DPC-like complexes.

Key words: dystrobrevin • dystrophin • synapse • postsynaptic density • astrocyte

DUCHENNE muscular dystrophy (DMD)¹ is the most common inherited muscle disease and one of the most severe. Although the primary cause of mortality in DMD patients is respiratory and cardiac failure brought on by muscle degeneration, other organ systems are implicated in the disease. Almost one third of DMD patients have mild mental retardation (Lidov, 1996). Dystrophin, the protein product of the DMD gene, is located at the postsynaptic membrane of cortical and Purkinje neurons, and is a component of the postsynaptic density (PSD; Lidov et al., 1990; Kim et al., 1992). These findings suggest that dystrophin plays an important role in the brain, where it may be involved in synapse function or in synaptic architecture.

In muscle, where the majority of studies have been conducted, dystrophin is associated with a protein complex,

the dystrophin-associated protein complex (DPC), located at the sarcolemma (Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991). In dystrophin-deficient muscle, this complex is disrupted, causing a severe reduction in the levels of the DPC at the membrane (Ervasti et al., 1990). The DPC can be subdivided into three distinct components, the dystroglycan complex, the sarcoglycan complex, and the cytoplasmic complex (reviewed by Blake and Davies, 1997).

The cytoplasmic complex is composed of α -dystrobrevin and the syntrophins (α , β 1, and β 2). In contrast to the sarcoglycans, which are only expressed in cardiac and skeletal muscle, components of the cytoplasmic complex and dystroglycan are found in many tissues, including the brain. α -Dystrobrevin is a dystrophin-related protein that binds directly to the COOH terminus of dystrophin and to the syntrophins (Blake et al., 1996; Nawrotzki et al., 1998; Peters et al., 1998). Although clearly important for muscle function, α -dystrobrevin is also thought to be involved in synaptic transmission at the neuromuscular junction (NMJ) and in intracellular signaling (Sanes et al., 1998; Grady et al., 1999). Similarly, members of the syntrophin family of proteins can bind to other signaling proteins, such as neuronal nitric oxide synthase (nNOS), voltage-gated sodium channels, stress-activated kinase 3, and mi-

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1. *Abbreviations used in this paper:* DMD, Duchenne muscular dystrophy; DPC, dystrophin-associated protein complex; LM, light membrane; NMDA, *N*-methyl *D*-aspartate; nNOS, neuronal nitric oxide synthase; PSD, postsynaptic density; SM, synaptic membrane.

croton tubule-associated serine/threonine kinase (Brenman et al., 1995; Gee et al., 1998; Hasegawa et al., 1999; Lumeng et al., 1999). These interactions suggest that the cytoplasmic components of the DPC may be directly involved in anchoring signaling proteins or ion channels to the sarcolemma in proximity to dystrophin.

Recently, a second member of the dystrobrevin family, β -dystrobrevin, was identified (Peters et al., 1997; Blake et al., 1998; Puca et al., 1998). The β -dystrobrevin gene encodes several alternatively spliced isoforms that are expressed predominantly in the brain and kidney, but not in muscle (Peters et al., 1997; Blake et al., 1998). In common with α -dystrobrevin, β -dystrobrevin also binds to dystrophin and syntrophin (Peters et al., 1997; Blake et al., 1998). Interestingly, in polarized epithelial cells, β -dystrobrevin, the dystrophin-related protein utrophin, and β 2-syntrophin form a complex at the basolateral surface (Kachinsky et al., 1999). These data suggest that β -dystrobrevin, along with other components of the DPC, is involved in the organization of specialized submembranous domains.

By analogy to the situation in muscle, a neuronal dystrophin-like complex may contribute to the etiology or neuropathology of cognitive impairment in DMD patients. However, whereas dystrophin is only expressed in neurons, several components of the DPC, such as dystroglycan and laminin-2, are associated with the glial/vascular interface (Jucker et al., 1996; Tian et al., 1996). Furthermore, utrophin and β 2-syntrophin, which are concentrated at the NMJ in muscle, are both associated with the cerebral microvasculature (Khurana et al., 1992; Lumeng et al., 1999). Thus, a dystrophin-like complex that is associated with microvascular glia exists in the brain. However, this complex does not contain dystrophin and is therefore not equivalent to the muscle described DPC.

To gain insight into the composition and location of DPC-like complexes in the brain, we have determined the distribution and molecular interactions of α - and β -dystrobrevin with dystrophin and its isoforms. We have shown that β -dystrobrevin is a neuronal, PSD-enriched, dystrophin-binding protein. By contrast, α -dystrobrevin-1 is found in glia, where it is primarily associated with the dystrophin isoform Dp71. Furthermore, both proteins assemble into distinct complexes with syntrophin in the brain that are maintained in the absence of dystrophin or Dp71. These data suggest that β -dystrobrevin is part of a neuronal DPC-like complex and may be involved in the compound phenotype of cognitive dysfunction in DMD patients. Our studies also highlight some fundamental differences between the molecular pathology of dystrophin deficiency in brain, compared with muscle.

Materials and Methods

Antibody Production

The following peptides were synthesized, coupled to keyhole limpet hemocyanin, and injected into New Zealand white rabbits: β 521, NH₂-CTGSPHTSPTHGGGRPM; pan-db, NH₂-CRVEHEQASQPTPEKAQQNP. Both antibodies were affinity-purified from the sodium sulphate precipitated IgG fraction of rabbit sera on immobilized peptide columns following the manufacturer's recommendations (Sulfolink, Pierce and Warriner). The antidystrophin antibody 2166 was raised against the last 17 amino acids of murine dystrophin and detects all

dystrophin isoforms. The antidystrophin antibody 2401 was raised against the alternatively spliced, hydrophobic COOH terminus of dystrophin, and detects Dp71. The antiutrophin polyclonal antibody, URD40, was raised against the distal rod domain of mouse utrophin and is preabsorbed against dystrophin. The SYN1351 antisyntrophin mAb was kindly supplied by Prof. Stan Froehner (University of North Carolina, Chapel Hill, NC; Froehner et al., 1987). The MANDRA1 antidystrophin mAb was kindly supplied by Prof. Glenn Morris (North East Wales Institute of Higher Education, Wrexham, UK; Nguyen et al., 1992). The PSD-95 and Munc-18 mAbs were purchased from Transduction Laboratories. The other dystrobrevin antisera, α 1CT-FP and β CT-FP, have been described previously (Blake et al., 1998; Nawrotzki et al., 1998).

Immunocytochemistry

Adult rats and mice were given an overdose of sodium pentobarbital (60 mg/kg i.p.) and the tissue was fixed by transcardiac perfusion with either 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), Bouin's fixative, or 70% ethanol in distilled water. The brains were removed, post-fixed overnight at 4°C, dehydrated, and paraffin-embedded. Sections were cut at 10–20 μ m in the transverse or sagittal planes and mounted on gelatin-coated slides. Sections were stained with rabbit antibodies, β 521 (1:200), α 1CT-FP (1:500), or 2166 (1:500), and mouse monoclonal antizebrin II (1:200) at the dilutions shown (Brochu et al., 1990). Indirect peroxidase immunocytochemistry was performed on the slide as described previously (Eisenman and Hawkes, 1993), with either rabbit anti-mouse IgG or goat anti-rabbit IgG conjugated to HRP as appropriate as the secondary antibody, and diaminobenzidine as the chromogen. Coverslips were applied with Permount. Images of sections and whole-mounts were digitally captured using a Sensys Camera (Optikon Corp. Ltd.) running under V for Windows. Montages were constructed using Adobe Photoshop 4.0. The images were cropped and corrected for brightness and contrast, but not otherwise manipulated.

For fluorescence microscopy, 10- μ m fixed sections were cut onto SuperFrost Plus slides (BDH). The sections were air dried and blocked in 10% normal donkey serum in TBS (150 mM NaCl, 50 mM Tris, pH 7.5) for 30 min. The sections were incubated with the primary antibody for 1 h, washed twice for 5 min in TBS, and then incubated with a 1:400 dilution of CY3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for another hour. Slides were washed twice for 5 min in TBS and mounted in Vectashield (Vector Laboratories) fluorescence medium containing 4',6-diamidino-2-phenylindole (DAPI). Slides were viewed with a Leica DMRE fluorescence microscope. For peptide blocking experiments, β 521 was preincubated with the immunizing peptide (25 μ M in TBS) or an unrelated peptide for 1 h at room temperature before application to the section.

Subcellular Fractionation and Synaptosome Preparation

Subcellular fractions, including synaptic membranes (SMs) and PSDs, were prepared from rat forebrain homogenates as described previously (Willmott et al., 1991; Mummery et al., 1996). 20 μ g of protein from each fraction was separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and incubated with an appropriate antibody. Crude synaptosomes were prepared from mouse brain following the method described by Blackstone et al. (1992). The crude synaptosomes were resuspended in PBS and stored at –20°C. Proteins were solubilized from synaptosome pellets by resuspending them in 1 M Tris HCl, pH 8.0, 100 mM NaHCO₃, pH 11.5, or 2% detergent (SDS, sodium deoxycholate, Tween-20, Triton X-100, N-lauroylsarcosine, digitonin) in PBS. After incubating the synaptosomes at 4°C for 30 min, the suspension was placed in a bench-top centrifuge and spun at 50,000 g_{av} for 30 min at 4°C. Soluble proteins extracted in the supernatant were removed and the resulting pellet was resuspended in the original volume of PBS. After the addition of an equal volume of 2 \times sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol) to each sample, the protein extracts were denatured and resolved by SDS-PAGE. The distribution of specific proteins contained in each fraction was determined by Western blotting with an appropriate antibody.

Immunoprecipitation

Tissue extracts from mouse and rat brain, and rat liver were prepared by

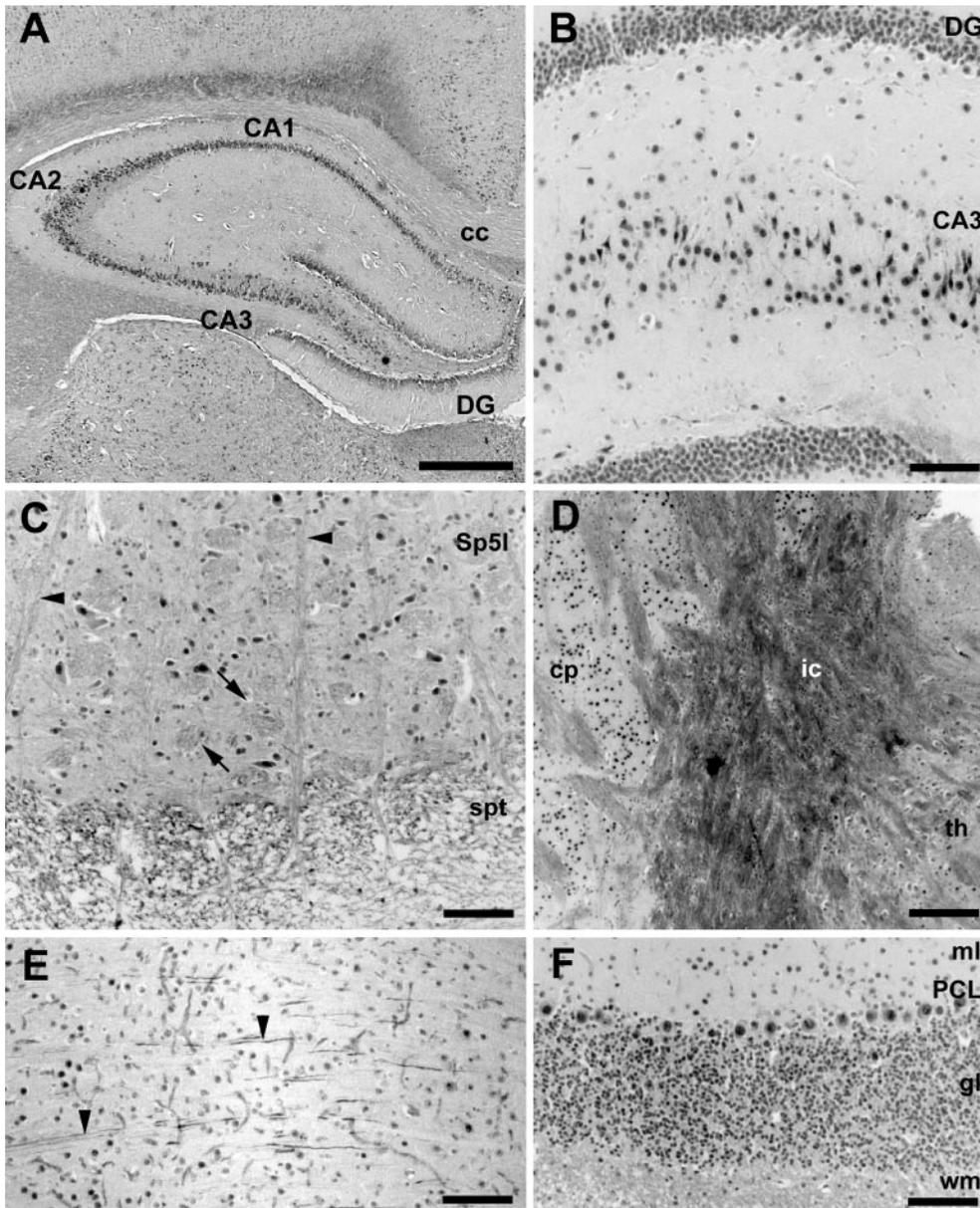


Figure 2. Immunolocalization of β -dystrobrevin in rat brain. The distribution of β -dystrobrevin immunoreactivity in transverse sections through the adult rat brain was determined with β 521. A, Hippocampus (low power). The reaction product is deposited in the CA1, CA2, CA3, and the dentate gyrus (DG). There is little staining of the corpus callosum (cc). Bar, 500 μ m. B, Hippocampus (midline to the left). Reaction product is found primarily in the neuronal somata. Staining is strongest in neurons of CA3, where the initial dendrite segments are also immunoreactive. The neurons of the dentate gyrus (DG) are also clearly labeled. Bar, 100 μ m. C, Reaction product is deposited in the interpolar spinal trigeminal nucleus (Sp5l), both in the nuclei of large neurons and in axon bundles cut longitudinally (arrowheads) and transversely (arrows) associated with the spinal trigeminal tract (spt). Bar, 100 μ m. D, Axonal immunoreactivity in the internal capsule (ic) and associated axon bundles in the caudate-putamen (cp) and thalamus (th). Bar, 100 μ m. E, Isocortex. Layer I is to the right. The nuclei of the pyramidal cells are prominently immunoreactive. In addition, reaction product is deposited in the pyramidal cell dendrites (dendritic fascicles are indicated by arrowheads). Bar, 200 μ m. F, Cerebellum. The somata and nuclei of all neuronal types are immunoreactive: the stellate and basket cells in the molecular layer (ml), the Purkinje cells in the Purkinje cell layer (PCL), and the granule cells in the granular layer (gl). In addition, the axons in the white matter (wm) are weakly stained. Bar, 100 μ m.

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β -Dystrobrevin immunoreactivity was detected in neurons. In the isocortex (Fig. 2 E), the reaction product is deposited most prominently in the somata (including the nuclei) and the dendrites of the pyramidal cells (Fig. 2 E). Other cortical neurons are also stained. Similarly, in the hippocampus (Fig. 2, A and B), immunoreactivity is most prominent in CA1, CA2, and CA3 neurons, where the reaction product is also found in the initial segments of dendrites. Neuronal somata are stained in other hippocampal regions as well. Somatal staining also characterizes the cerebellum (Fig. 2 F), where the Purkinje cells, the molecular layer interneurons, and the granule cells are immunoreactive. In all cases, there is also weak axonal staining. Examples are shown for the brainstem, where strong immunore-

activity is detected in axon fascicles associated with the spinal trigeminal tract (Fig. 2 E) and in the internal capsule (Fig. 2 D).

Immunolocalization of α -Dystrobrevin-1 in the Brain

The α 1CT-FP antibody was used to locate α -dystrobrevin-1 in brain sections. As for β -dystrobrevin, there were no significant species differences, and the same distribution was seen with all methods of fixation used. α -Dystrobrevin-1 is expressed by perivascular astrocytes in a pattern that is reminiscent of utrophin immunoreactivity (Khurana et al., 1992). Seen at low magnification, reaction product is deposited throughout the brain (Fig. 3 A). In the isocortex,

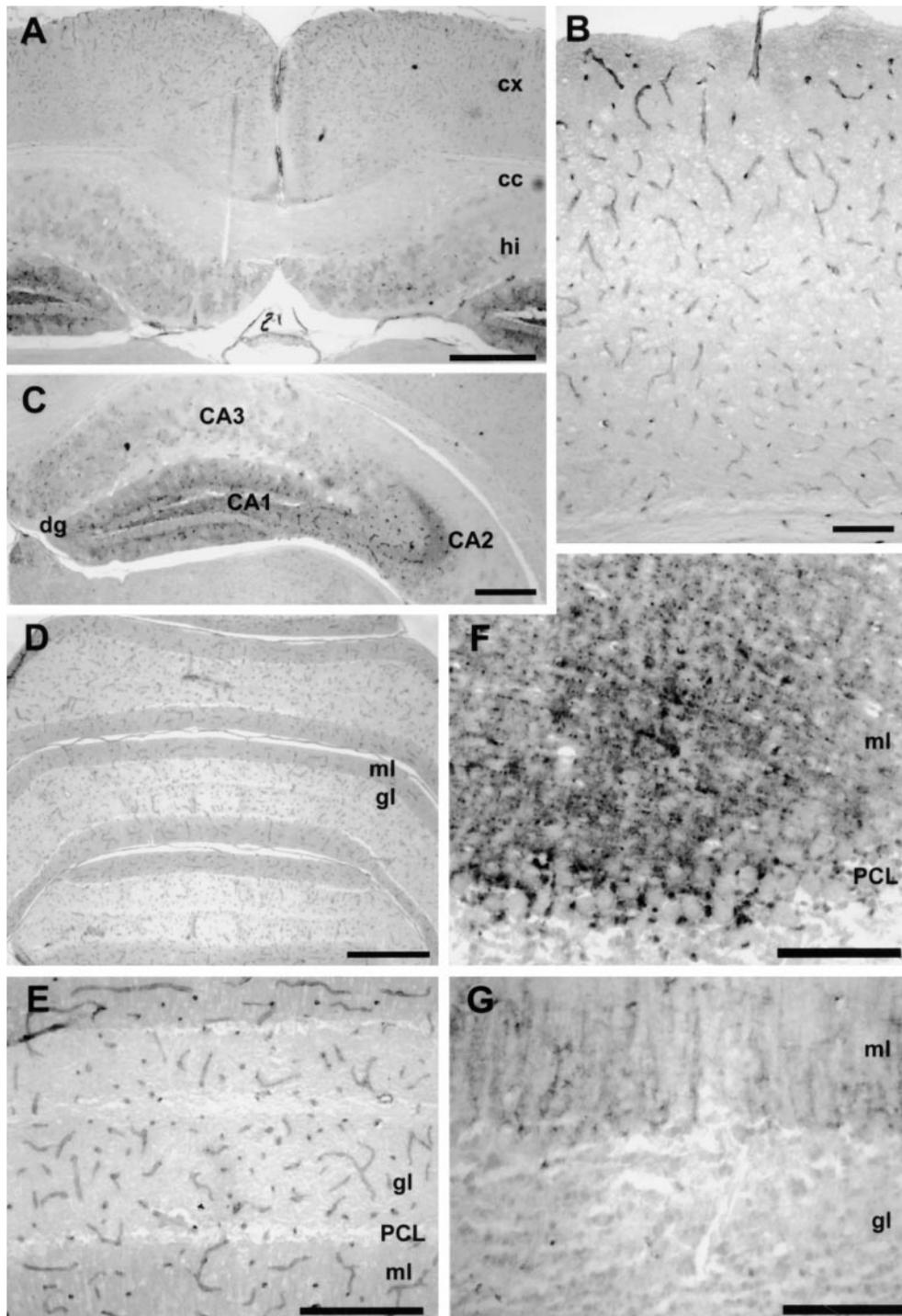


Figure 3. Immunolocalization of α -dystrobrevin-1 in rat and mouse brain. The distribution of α -dystrobrevin-1 immunoreactivity in transverse sections through the adult rat (A–F) and mouse brain (G) was determined with α 1CT-FP. A, Low-power view through the fore-brain. Reaction product is deposited in the cortex (cx) and hippocampus (hi), but little immunoreactivity is detected in the corpus callosum (cc). Bar, 1 mm. B, In the isocortex, the reaction product is deposited primarily in blood vessels. Bar, 200 μ m. C, Hippocampus (midline to the left). Reaction product is found primarily in glial cells. There are prominent regional differences in staining intensity. Most reaction product is deposited in the dentate gyrus (dg) and CA2/CA3. There is much less immunoreactivity in CA1. Bar, 100 μ m. D, Cerebellum. At low magnification, immunoreactivity is clearly laminar, with moderate staining of the molecular layer (ml), speckled staining in the granular layer (gl), and little, if any, immunoreactivity in the white matter tracts. Bar, 500 μ m. E, Cerebellar reaction product in the granular layer is confined to the blood vessels. Vascular staining is also present in the molecular layer. There is no neuronal staining, e.g., granule cells in the granular layer or Purkinje cells in the Purkinje cell layer (PCL). Bar, 200 μ m. F, Cerebellar immunoreactivity in the molecular layer is concentrated in the small somata of the Bergmann glia, scattered among the unstained Purkinje cell somata in the Purkinje cell layer. Bar, 100 μ m. G, Cerebellar immunoreactivity in the molecular layer of the adult mouse. The distribution of immunoreactivity is similar to that in the rat, except that the radial nature of the Bergmann glial fibers in the molecular layer is more easily recognized. Bar, 100 μ m.

layer, and is also expressed diffusely throughout the molecular layer in the Bergmann radial glial processes. Bar, 100 μ m. G, Cerebellar immunoreactivity in the molecular layer of the adult mouse. The distribution of immunoreactivity is similar to that in the rat, except that the radial nature of the Bergmann glial fibers in the molecular layer is more easily recognized. Bar, 100 μ m.

the only prominent staining is associated with the vasculature (Fig. 3 B). In the hippocampus, in addition to staining of blood vessels, there is prominent astroglial immunoreactivity (Fig. 3 C). There are also conspicuous regional differences in staining intensity. Most reaction product is deposited in the dentate gyrus and CA2/CA3, with much less immunoreactivity in CA1. A similar combination of peri-

vascular and astroglial immunoreactivity is seen in the cerebellum (Fig. 3, D–G). At low magnification, α -dystrobrevin-1 immunoreactivity is clearly laminar, with moderate staining of the molecular layer, speckled staining in the granular layer, and little, if any, immunoreactivity in the white matter tracts (Fig. 3 D). No regional differences are apparent in the cerebellum. The granular layer immunore-

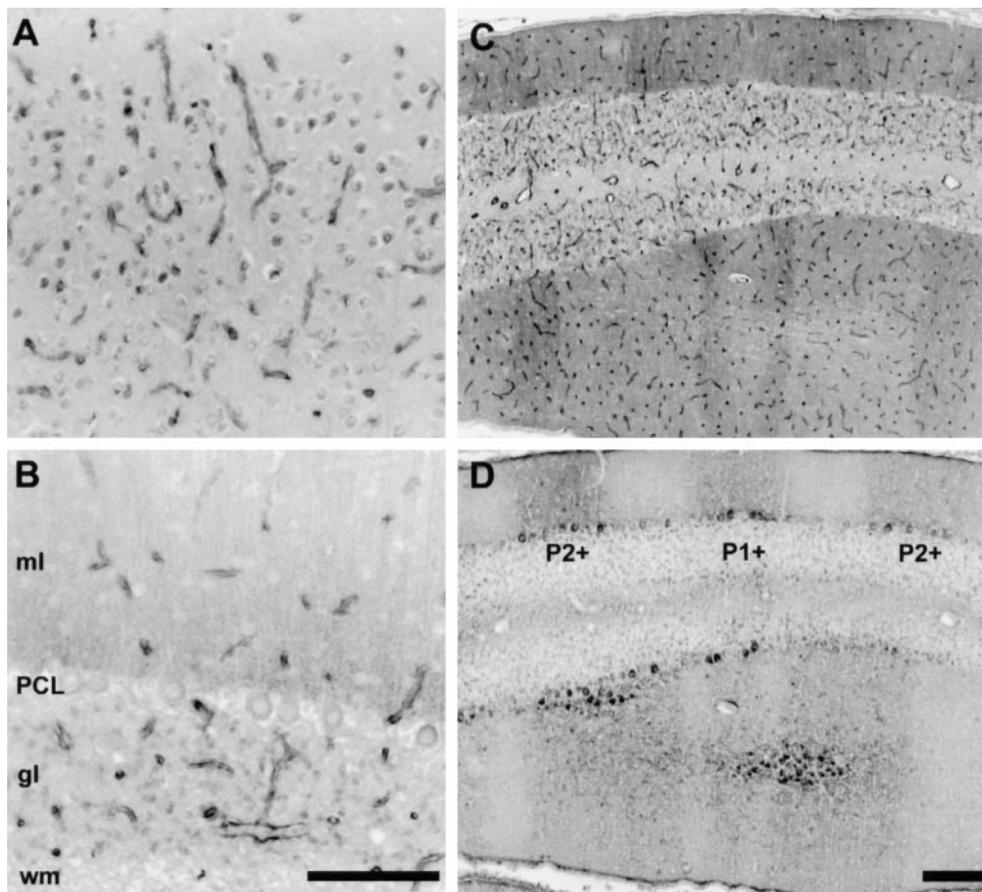


Figure 4. Immunolocalization of dystrophin and its isoforms in rat brain. The distribution of dystrophin immunoreactivity in transverse sections was determined with the 2166 antibody. A, In the isocortex, neuronal somata and blood vessels are labeled. B, In the cerebellar cortex, Purkinje cell somata are labeled in the Purkinje cell layer (PCL), and their dendrites are seen in the molecular layer (ml). There is weaker granule cell immunoreactivity in the granular layer (gl) and little or no immunoreactivity in the white matter (wm). Bar (A and B), 100 μ m. C, Cerebellar cortex. At low magnification, the labeling of Purkinje cell dendrites is clearly non-uniform and reveals alternating parasagittal bands of Purkinje cell dendrites (seen here flanking the midline in lobule VIII). D, Serial section labeled with the Purkinje cell band marker, antizebrin II. The P1+ band at the midline and the P2+ bands laterally are labeled. The antizebrin II-negative Purkinje cell bands preferentially express dystrophin. Bar (C and D), 200 μ m.

activity is confined to the vasculature, and there is no staining of granule cells (Fig. 3 E). At higher magnification, the reaction product in the molecular layer is concentrated in the Bergmann astroglial cells, where both the somata (Fig. 3 F) and the radial glial processes (Fig. 3, F and G) are stained. The Purkinje cells were not immunoreactive.

Immunolocalization of Dystrophin and Its Isoforms in the Brain

The two major dystrophin isoforms present in brain are dystrophin and Dp71. In addition to these products, Dp140 is also found as a minor component in brain extracts. To investigate the location of dystrophin-immunoreactive proteins in the brain, sections of rat and mouse brain were stained with the antibody 2166 that detects all dystrophin isoforms. This antibody gave a complex staining pattern attributable to the presence of three different proteins (Fig. 4). In the isocortex, 2166 labeled neuronal somata and blood vessels (Fig. 4 A). In the cerebellar cortex, Purkinje cell somata are labeled, along with dendrites in the molecular layer (Fig. 4 B). There is weak labeling of granule cells and little, if any, labeling in the white matter (Fig. 4 B). A novel feature of the dystrophin isoform expression pattern is that the levels of staining intensity vary in a consistent fashion between Purkinje cells. At low mag-

nification, the labeling of Purkinje cell dendrites is nonuniform and appears as a symmetrical and reproducible parasagittal banded appearance when viewed in the transverse plane (Fig. 4 C). This distribution is reminiscent of the pattern revealed by immunostaining for the well-known Purkinje cell band marker zebrin II. When the pattern of dystrophin isoform expression is compared with that of zebrin II (Fig. 4 D; Brochu et al., 1990; Eisenman and Hawkes, 1993; Ahn et al., 1994). It is evident that the Purkinje cells which preferentially express dystrophin are zebrin II negative. This pattern of dystrophin immunoreactivity in cerebellar striations is similar to the high microtubule-associated protein (MAP) 1A expressing subset (Touri et al., 1996).

Distribution of the Dystrobrevins in Subcellular and Synaptic Fractionations

The differential location of β - and α -dystrobrevin-1 in neurons and glia, respectively, suggests that the dystrobrevins could be independent, cell type-restricted ligands for dystrophin and its isoforms in the brain. To test this hypothesis, we examined the subcellular distribution of the dystrobrevins and the molecular organization of dystrobrevin-containing protein complexes. The subcellular distribution of the dystrobrevins was determined by immuno-

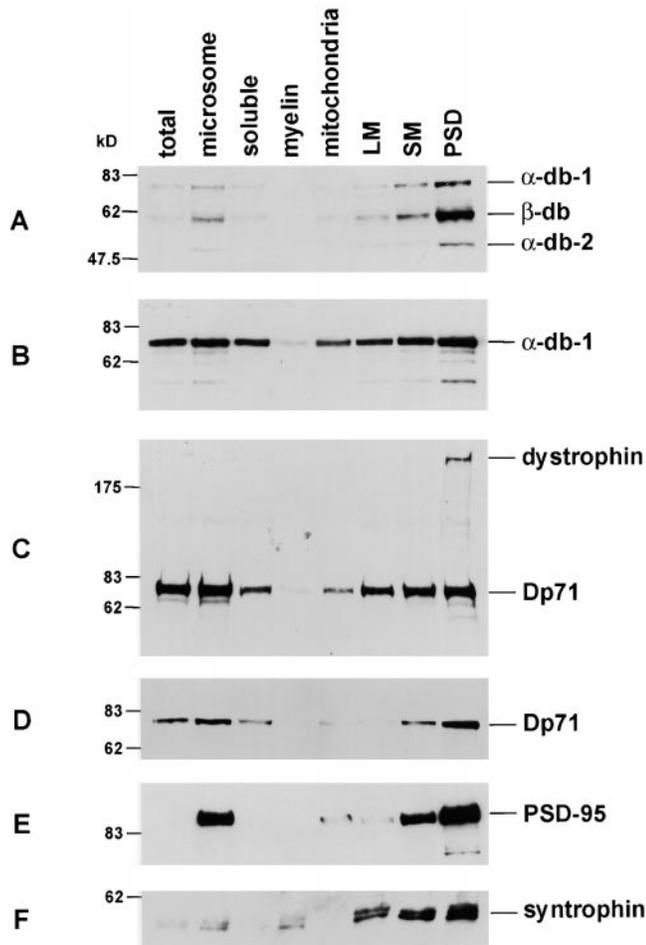


Figure 5. Subcellular fractionation of the dystrophin-related proteins in the brain. The enrichment of β -dystrobrevin (A), α -dystrobrevin-1 (B), dystrophin isoforms (C and D), PSD-95 (E), and syntrophin (F) in a range of subcellular fractions prepared from rat forebrain, including SMs and postsynaptic densities, was determined by Western blotting. The antibody β CT-FP detects β -dystrobrevin and α -dystrobrevin-1 and -2 (A). β -Dystrobrevin is highly enriched in the PSD fraction, whereas α -dystrobrevin-1 is not enriched (A and B). Although only a minor component in brain extracts, a band corresponding to α -dystrobrevin-2 is also enriched in the PSD (A). Dystrophin and Dp71, detected with the antibody 2166, are also detected in PSDs (C). While Dp71 is found in a wide range of fractions, the majority of dystrophin is found in the PSD fraction. The enrichment of Dp71 in the PSD fraction was confirmed with the MANDRA1 mAb (D). E, Shows the enrichment of PSD-95 in the PSD fraction. Similarly, syntrophin detected with the pansyntrophin mAb, SYN1351, is also PSD-enriched (F). The positions of the molecular weight standards are indicated.

blotting a range of subcellular fractions, including SMs and PSDs prepared from rat forebrain (Fig. 5). Immunoblots were incubated with a panel of antibodies specific for the dystrobrevins, dystrophin and its isoforms, the syntrophins, and the PSD-enriched protein, PSD-95 (Fig. 5). β -Dystrobrevin was found to be highly enriched in the PSD fraction, when compared with the light membrane (LM) and SM fractions (Fig. 5 A). The LM fraction comprises membrane fragments primarily of nonsynaptic origin. This result was confirmed using the β -dystrobrevin-

specific antibody, β 521 (data not shown). By contrast, α -dystrobrevin-1 was found in most fractions and was only moderately enriched in the PSD fraction, when compared with the LM and SM fractions (Fig. 5, A and B). In control experiments, the enrichment of the PSD protein, PSD-95, was determined in the same subcellular fractions. As expected, PSD-95 was highly enriched in the PSD fraction, compared with the LM and SM fractions (Fig. 5 E; Cho et al., 1992).

To determine whether the potential dystrobrevin-binding proteins were PSD-enriched, duplicate blots were incubated with antidystrophin and antisyntrophin antibodies. Dystrophin and Dp71 were enriched in the PSD fraction (Fig. 5 C). Furthermore, Dp140, which is thought to be located in perivascular astrocytes (Lidov et al., 1995), was not PSD-enriched. The enrichment of Dp71 in the PSD was confirmed with the MANDRA1 mAb that is raised against the COOH terminus of dystrophin (Fig. 5 D). On longer exposures, dystrophin was detected in the PSD preparations with MANDRA1 (data not shown). Also, the syntrophins were found to be PSD-enriched (Fig. 5 F). The SYN1351 mAb detects a doublet of proteins of \sim 59-kD that could correspond to the two syntrophin isoforms (α and β) found in brain (Górecki et al., 1997). Thus, several proteins that are potentially associated with β -dystrobrevin are PSD-enriched.

To examine the biochemical association of the dystrobrevins with SMs in brain, synaptosomes were prepared and solubilized with a panel of detergents and buffers. The experiments were not carried out on PSDs due to the highly insoluble nature of this material. The distribution of the dystrobrevins in subcellular fractions was determined by immunoblotting. Interestingly, the dystrobrevins were found in most fractions, including the soluble fraction and the nuclear pellet (Fig. 6, upper panel), whereas the major-

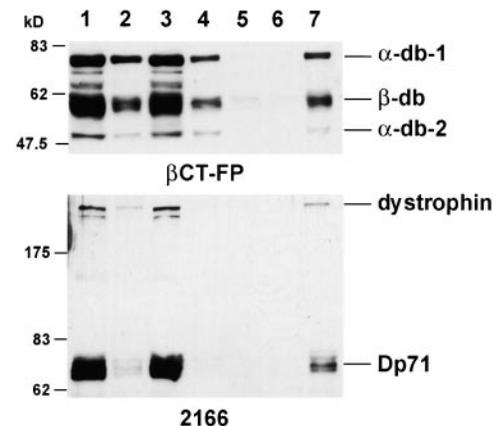


Figure 6. Distribution of the dystrobrevins and dystrophin in subcellular fractions. Upper panel, Western blot of brain extracts incubated with β CT-FP, showing the distribution of β -dystrobrevin and α -dystrobrevin-1 and -2 in the protein fractions. Lower panel, Western blot of brain extracts incubated with the antibody 2166, showing the distribution of dystrophin and Dp71 in the fractions. Lane 1, total homogenate; lane 2, nuclear pellet; lane 3, postnuclear supernatant; lane 4, soluble; lane 5, wash 1; lane 6, wash 2; and lane 7, crude synaptosomes. The positions of the molecular weight standards are indicated.

Table I. Solubilization of Proteins from Crude Synaptosomes

Extraction buffer	β -Dystrobrevin	α -Dystrobrevin-1	Dystrophin	Dp71	PSD-95
PBS	-	-	-	-	-
0.1 M NaHCO ₃ , pH 11.5	-	-	-	-	-
1 M Tris, pH 8.0	-	-	-	-	-
2% SDS	++	++	++	++	++
2% deoxycholate	-	+	-	-	-
2% Tween-20	-	-	-	-	-
2% Triton X-100	-	-	-	-	-
2% <i>N</i> -lauroylsarcosine	++	++	++	++	+/-
2% digitonin	-	-	-	-	-

Soluble and insoluble proteins extracted from crude synaptosomes were analyzed by Western blotting. The amount of protein extracted from the synaptosomes was scored arbitrarily as follows: ++, fully soluble; +, partially soluble; +/-, weakly soluble; and -, insoluble.

ity of dystrophin and Dp71 was found in the crude membrane and synaptosomal fractions (Fig. 6, lower panel). Most proteins that are PSD-enriched are only solubilized using high concentrations of ionic detergents (Kennedy, 1997). To determine the extractability of the dystrobrevins from crude synaptosomes, washed crude synaptosomes were resuspended in a variety of buffers or in PBS containing different detergents (Table I). α - and β -dystrobrevin, dystrophin, and Dp71 were completely solubilized in 2% SDS and 2% *N*-lauroylsarcosine. However, α -dystrobrevin-1 was partially solubilized in 2% deoxycholic acid also, whereas β -dystrobrevin, dystrophin, and Dp71 were

insoluble in this detergent. These data suggest that β -dystrobrevin and dystrophin are tightly associated with cytoskeletal structures, predominantly the PSD. In control experiments, the extractability of PSD-95 was also determined. PSD-95 was only extracted in 2% SDS (Table I).

Dystrobrevin Interactions with Dystrophin, Dp71, and Utrophin

To examine the interactions between β -dystrobrevin and the dystrophin family of proteins in brain, reciprocal immunoprecipitations were performed on rat brain and liver

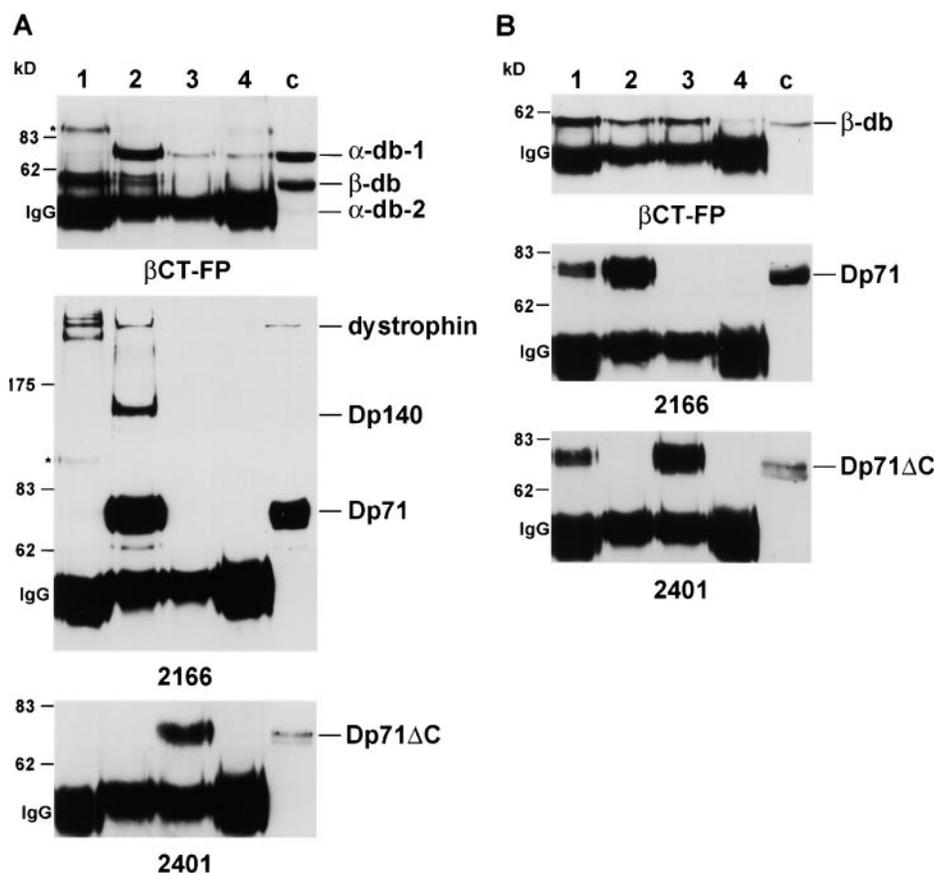


Figure 7. Association of β -dystrobrevin with dystrophin in the brain. RIPA extracts of rat brain (A) and rat liver (B) were immunoprecipitated with β 521 (lane 1), 2166 (lane 2), 2401 (lane 3), and URD40 (lane 4). The coimmunoprecipitated proteins were detected with the antibody listed below each panel. In brain, β -dystrobrevin is only immunoprecipitated by 2166 and the cognate antibody (A, upper panel). Reciprocal immunoprecipitation by β 521 detected with antibody 2166 reveals that dystrophin, and not Dp71 and Dp140, is the only protein that coimmunoprecipitates with β -dystrobrevin (A, middle panel). β -dystrobrevin is not immunoprecipitated by 2401 or URD40 (A, lower panel). In control experiments on rat liver that demonstrate the efficacy of each antibody for immunoprecipitation, β -dystrobrevin is immunoprecipitated by 2166, 2401, and URD40 (B, upper panel). Reciprocal immunoprecipitations confirm the association of β -dystrobrevin with Dp71 (B, middle panel) and Dp71 Δ C (B, lower panel) in rat liver. The loading control (c) in each experiment, RIPA brain extract (A) or RIPA liver extract (B), is equivalent to 10% of the input

protein from each immunoprecipitation. The 100-kD protein in lane 1 (* in the upper and middle panels) is an IgG cross-reactive protein and is detected with the secondary antibody alone. The rabbit IgG heavy chain and the positions of the molecular weight standards are indicated.

using a panel of polyclonal antibodies. For these experiments, rat tissue was used because rat β -dystrobrevin has a slightly higher relative mobility on SDS-PAGE, compared with mouse β -dystrobrevin. This enables a better separation of β -dystrobrevin from the rabbit IgG heavy chain. Rat liver was used as a control tissue because it is an abundant source of β -dystrobrevin and can therefore be used to check the efficacy of the antibodies used for immunoprecipitation. In brain, β -dystrobrevin was only coimmunoprecipitated with the antidystrophin antibody 2166 (Fig. 7 A, upper panel). In reciprocal experiments, β 521 coimmunoprecipitated dystrophin (Fig. 7 A, middle panel). On long exposures, Dp71 was also detected, albeit weakly, in β -dystrobrevin immunoprecipitates (Fig. 7 A, middle panel). Although Dp140 was immunoprecipitated by 2166, no Dp140 was coimmunoprecipitated with β -dystrobrevin. Furthermore, 2401 and URD40 failed to coprecipitate β -dystrobrevin (Fig. 7 A, lower panel). In the liver, β -dystrobrevin coimmunoprecipitated with Dp71, Dp71 Δ C, and utrophin (Fig. 7 B). These data show that β -dystrobrevin is specifically associated with dystrophin in the brain, but can also bind to Dp71 or utrophin in the liver.

To determine the composition of α -dystrobrevin-1-containing protein complexes in the brain, α -dystrobrevin-1

was immunoprecipitated from rat brain with a panel of polyclonal antibodies (Fig. 8). α -Dystrobrevin-1 was coimmunoprecipitated by 2166 and URD40 (Fig. 8, upper panel). However, the relative amounts of α -dystrobrevin-1 immunoprecipitated by each antibody were very different, suggesting that the major complex formed is between α -dystrobrevin-1 and Dp71. Reciprocal immunoprecipitations confirmed the association of α -dystrobrevin-1 with Dp71 (Fig. 8, middle panel) and utrophin (Fig. 8, upper panel). No α -dystrobrevin-1 was immunoprecipitated by β 521, showing that β -dystrobrevin and α -dystrobrevin-1 are in different protein complexes. Furthermore, Dp140 and dystrophin failed to immunoprecipitate with α -dystrobrevin-1 or utrophin (data not shown).

Dystrobrevin Interactions in Dystrophin-deficient Mice

The syntrophin family of proteins have been shown to bind directly to α -dystrobrevin in muscle (Ahn and Kunkel, 1995). To investigate this interaction in brain, we examined the association of the dystrobrevins with syntrophin in the presence and absence of dystrophin and Dp71. The pansyntrophin antibody, SYN1351, was used to immunoprecipitate syntrophin-containing protein complexes from RIPA-extracted crude membranes prepared from the brains of normal C57 mice, dystrophin-deficient *mdx* mice, and *mdx*^{3Cv} mice, which lack dystrophin and all the COOH-terminal dystrophin isoforms (Cox et al., 1993). The two predominant dystrobrevin isoforms present in brain, α -dystrobrevin-1, and β -dystrobrevin are precipitated with SYN1351 (Fig. 9 A, upper panel). Furthermore, a protein corresponding to α -dystrobrevin-2 was also precipitated by SYN1351. The amount of protein precipitated from each mouse strain appeared to be similar, indicating that the interaction between the dystrobrevins and syntrophin was unperturbed by the absence of dystrophin and Dp71 (Fig. 9 A, middle panels). As expected, no dystrophin cross-reactive proteins were immunoprecipitated with SYN1351 from the brains of *mdx*^{3Cv} mice (Fig. 9 A, middle panel). To exclude the possibility that nonspecific protein precipitation had occurred, duplicate Western blots were incubated with an mAb raised against the synaptic vesicle protein, Munc18. Munc18 was not detected in immunoprecipitates from any of the mouse strains, indicating that the SYN1351 antibody specifically immunoprecipitated dystrobrevin and dystrophin-containing complexes (Fig. 9 A, lower panel). Furthermore, no apparent difference in dystrobrevin immunoreactivity in the brains of normal mice, compared with dystrophin-deficient mice, was found (data not shown). In control experiments, we investigated the association of syntrophin with α -dystrobrevin in skeletal muscle extracts prepared from normal and *mdx* mice. By contrast to the situation in brain, the absence of dystrophin results in a dramatic reduction in the amount of α -dystrobrevin-1 and -2 associated with syntrophin (Fig. 9 B). These results are consistent with the reduction of α -dystrobrevin immunoreactivity at the sarcolemma of dystrophin-deficient muscle (Nawrotzki et al., 1998). Thus, the lack of dystrophin in muscle affects the association of α -dystrobrevin and syntrophin, whereas in the brain, this interaction is apparently unaltered by the absence of dystrophin or Dp71.

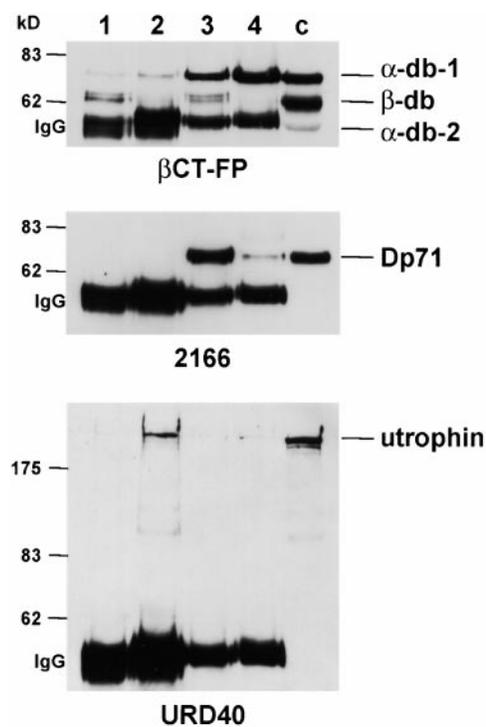


Figure 8. Association of α -dystrobrevin-1 with Dp71 and utrophin in the brain. RIPA extracts of rat brain were immunoprecipitated with β 521 (lane 1), URD40 (lane 2), 2166 (lane 3), α 1CT-FP (lane 4). Immunoprecipitated proteins were analyzed by Western blotting with β CT-FP (upper panel), 2166 (middle panel), and URD40 (lower panel). In brain extracts, α -dystrobrevin-1 coimmunoprecipitates predominantly with Dp71, but also with utrophin. Reciprocal coimmunoprecipitations confirm these associations. The loading control (c) in each experiment was RIPA brain extract, and is equivalent to 10% of the input protein from each immunoprecipitation. The rabbit IgG heavy chain and the positions of the molecular weight standards are indicated.

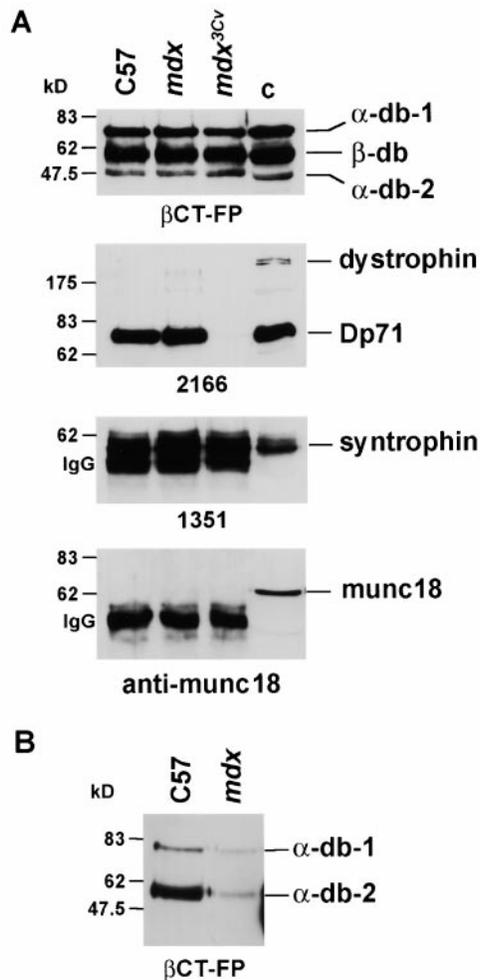


Figure 9. The dystrobrevin–syntrophin complex. **A**, Association of syntrophin with the dystrobrevins in the brain RIPA extracts prepared from C57, *mdx*, and *mdx*^{3Cv} mouse brains were immunoprecipitated with the SYN1351 pansyntrophin mAb. The coimmunoprecipitated proteins were Western blotted and incubated with the following antibodies: βCT-FP (upper panel); 2166 (second panel); SYN1351 (third panel); and Munc18 (lower panel). The RIPA-soluble brain extract that was used for immunoprecipitation was included as a control (c). Equivalent amounts of the three dystrobrevin isoforms are coimmunoprecipitated with the SYN1351 antibody in extracts from the three mouse strains (upper panel). Dp71 is coimmunoprecipitated by SYN1351 in C57 and *mdx* extracts (second panel). The levels of syntrophin precipitated from each extract are shown to be equivalent (third panel). Munc18 is not present in SYN1351 immunoprecipitates (lower panel). The rabbit IgG heavy chain and the positions of the molecular weight standards are indicated. **B**, Association of syntrophin with α-dystrobrevin-1 and -2 in muscle. RIPA extracts prepared from C57 and *mdx* mouse muscle were immunoprecipitated with the SYN1351 antibody. The coimmunoprecipitated proteins were Western blotted and incubated with βCT-FP. α-Dystrobrevin-1 and -2 are precipitated with the SYN1351 mAb. The amount of α-dystrobrevin-1 and -2 precipitated from *mdx* muscle are significantly less than the levels precipitated from C57 muscle. The positions of the molecular weight standards are indicated.

Discussion

In this paper, we have presented several lines of evidence showing that β-dystrobrevin is a component of a DPC in neurons. To the best of our knowledge, this is the first example of a protein that is directly and specifically associated with dystrophin in the brain. We have shown that the closest relative of β-dystrobrevin, α-dystrobrevin-1, in common with several other components of the DPC, is associated with perivascular astrocytes and other glial cells. Thus, despite extensive sequence homology, both proteins are differentially distributed in the brain, where they form distinct protein complexes.

β-Dystrobrevin Localization in the Brain

In the brain, β-dystrobrevin is found in neurons in the cortex, hippocampus, and cerebellum, where it is associated with neuronal somata, dendrites, and nuclei (Fig. 2). This location is similar to that described for dystrophin (Fig. 4; Lidov et al., 1990, 1993). The location of β-dystrobrevin and dystrophin in the same types of neurons supports our proposal that these proteins form part of a neuronal DPC-like complex. However, there are some distinct differences between the neuronal locations of β-dystrobrevin and dystrophin. For example, in the cerebellum, β-dystrobrevin is predominantly expressed in granule cells and in Purkinje cell somata (Fig. 2 F). By contrast, dystrophin immunoreactivity was not found in granule cell neurons, but predominated in the Purkinje cell somata and dendrites and in perivascular astrocytes (Fig. 4 B). Interestingly, β-dystrobrevin is found at sites that are not reported to express dystroglycan, such as granule cells and axons (Blake, D.J., unpublished results; Tian et al., 1996). Since dystroglycan is a bind partner for dystrophin and its isoforms, these data could suggest that a proportion of β-dystrobrevin is not associated with DPC-like complexes in the brain.

The association of β-dystrobrevin with some neuronal nuclei reflects this theme (Fig 2). This result is supported by the presence of significant amounts of β-dystrobrevin in the nuclear pellet, compared with the content of Dp71 and dystrophin in the same fraction (Fig. 6). β-dystrobrevin lacks an obvious nuclear localization signal, but does have some sequence features typical of nuclear proteins, such as the zinc finger-like ZZ domain (Ponting et al., 1996). It is interesting to speculate that β-dystrobrevin may translocate from the neuronal membrane to the nucleus. This mechanism has been described for a number of proteins that are involved in the acquisition of long-term memory (Abel and Kandel, 1998; Deisseroth et al., 1998). A similar mechanism has been demonstrated for β-catenin, a protein that plays a dual role in cadherin-mediated cell adhesion at specialized submembranous sites and in Wnt-signaling in the nucleus. Like β-dystrobrevin, β-catenin is found at the membrane, in the cytoplasm, and nucleus, but has no obvious nuclear import signal (Fagotto et al., 1998; Wodarz and Nusse, 1998).

While it is difficult to envisage a role for members of the DPC in the nucleus, it should be emphasized that the recently characterized syntrophin-binding protein, SAST (syntrophin-associated serine/threonine kinase), is also found in the nuclei of hippocampal pyramidal neurons (Lumeng et al., 1999). Our data and the data of Lumeng

and colleagues raise the possibility that DPC-like complexes may be present in the nucleus. Clearly, establishing a role for these proteins in the nucleus warrants further investigation.

A Role for the Neuronal DPC-like Complex in Brain Function?

We have shown that β -dystrobrevin colocalizes and coimmunoprecipitates with dystrophin in neurons. Furthermore, β -dystrobrevin, dystrophin, and syntrophin are PSD-enriched. These data strongly suggest that β -dystrobrevin is a neuronally expressed dystrophin-associated protein. Additionally, β -dystrobrevin coimmunoprecipitates with syntrophin (Fig. 9) and interacts with the syntrophin family of proteins in the yeast two-hybrid system (Benson, M.A., and D.J. Blake, unpublished results). Our data suggest that the DPC-like complex in neurons is composed of dystrophin, β -dystrobrevin, and syntrophin (Fig. 10 A). Therefore, by analogy to the situation in muscle, this complex may be involved in the etiology of cognitive impairment in DMD patients. The molecular identity of other components of this complex is currently unknown. However, a wealth of circumstantial evidence suggests that nNOS may also be associated with the dystrophin- β -dystrobrevin complex in neurons.

In the brain, α -syntrophin interacts with nNOS through reciprocal PDZ domains (Brenman et al., 1995, 1996; Hashida-Okumura et al., 1999; Hillier et al., 1999). Furthermore, nNOS also binds to the PSD proteins, PSD-93 and PSD-95, which are involved in *N*-methyl D-aspartate (NMDA) receptor clustering (Brenman et al., 1996). This interaction places nNOS in proximity to the NMDA receptor at the postsynaptic membrane of excitatory synapses (Kornau et al., 1995; Niethammer et al., 1996). Our finding that β -dystrobrevin and the syntrophins are PSD-enriched and present in a complex offers a separate mechanism to locate nNOS to the postsynaptic membrane. Therefore, it is a distinct possibility that the DPC-like complex at the neuronal membrane contains nNOS bound to syntrophin, β -dystrobrevin, and dystrophin (Fig. 10 A). This interaction, coupled with the role of nitric oxide in the calcium-dependent processes of neurotoxicity, could render dystrophin-deficient neurons more susceptible to metabolic or physiological insults (Christopherson and Brecht, 1997). This hypothesis is supported by the findings that dystrophin-deficient neurons have enhanced susceptibility to hypoxia-induced loss of synaptic transmission (Mehler et al., 1992) and have increased intracellular calcium (Hopf and Steinhardt, 1992). Hypoxia and hyperexcitation cause parallel increases in intracellular calcium in neurons that can ultimately lead to cell death. Consistent with these ideas, an increase in neuronal cell loss has been described in some patients with DMD (Jagadha and Becker, 1988).

α -Dystrobrevin-1 Localization and Association in the Brain

We have shown that α -dystrobrevin-1 is found in perivascular astrocytes and in Bergmann glia of the cerebellum (Fig. 3). The pattern of α -dystrobrevin-1 immunoreactivity in the brain is reminiscent of utrophin that was shown to

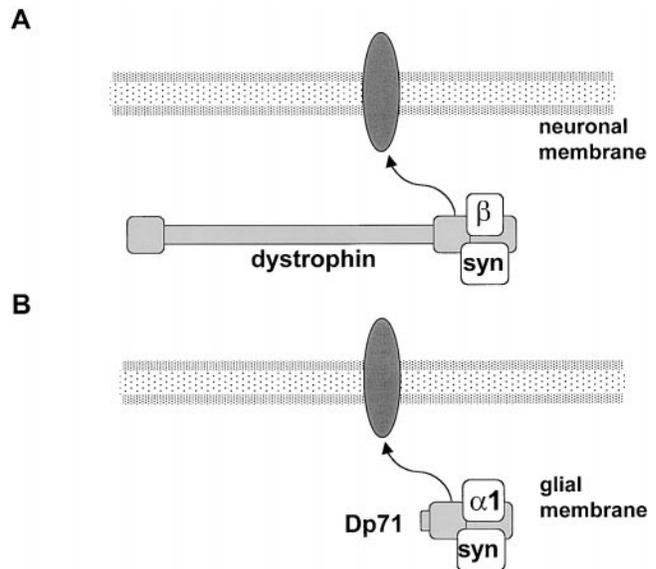


Figure 10. Dystrobrevin containing complexes in the brain. Hypothetical model showing the organization of DPC-like complexes in neurons (A) and glia (B). In neurons, β -dystrobrevin is associated with dystrophin and syntrophin. In glia, such as perivascular astrocytes, α -dystrobrevin-1 is found in a complex with Dp71 and syntrophin. In both instances, we propose that there is a transmembrane binding partner (filled oval) for dystrophin and Dp71, similar to the dystroglycan-dystrophin complex in muscle. For clarity, the minor complexes containing β -dystrobrevin and Dp71 and α -dystrobrevin-1 and utrophin are not shown.

be associated with the cerebral microvasculature and, in particular, the foot processes of perivascular astrocytes (Khurana et al., 1992). Other proteins known to be associated with the DPC, such as dystroglycan (Tian et al., 1996), laminin-2 (Jucker et al., 1996), the dystrophin isoform Dp140 (Lidov et al., 1995), and agrin (Barber and Lieth, 1997) are expressed in perivascular regions of the brain. The accumulation of α -dystrobrevin-1 and the DPC components at these sites may play a role in the pathogenesis of viral infections of the central nervous system since α -dystroglycan has been shown to be a receptor for several arenaviruses (Cao et al., 1998). Thus, DPC-like complexes in perivascular astrocytes may form part of the permeability barrier between the circulatory system and the brain.

In astrocytes, α -dystrobrevin-1 is predominantly associated with Dp71 and syntrophin. However, a minor complex is also formed between α -dystrobrevin-1 and utrophin. Therefore, it is possible that different DPC-like complexes are maintained within glial cells. It is noteworthy that Dp140, a protein that is expressed in glial cells associated with brain vasculature (Lidov et al., 1995), does not copurify with α -dystrobrevin-1 in brain extracts (Fig. 8). Since α -dystrobrevin-1 is only found in glial cells, a large proportion of Dp71 must also be expressed by glia, accounting for the perivascular staining of the antidystrophin antibody 2166 in the brain (Fig. 5). Thus, the DPC-like complex containing α -dystrobrevin-1 in glia is predominantly composed of α -dystrobrevin-1, Dp71, and syntrophin (Fig. 10 B). There is also some evidence that

Dp71 may be present in neurons, especially in the dentate gyrus (Górecki et al., 1998). It is possible that neuronally derived Dp71 is associated with β -dystrobrevin because a small amount of Dp71 was detected amongst the proteins immunoprecipitated by β 521 (Fig. 8). Furthermore, we have shown that Dp71 is PSD-enriched (Fig. 5, C and D). However, some glial-derived proteins, such as α -dystrobrevin-1, are clearly present in PSD preparations. PSDs are known to absorb proteins of a nonneuronal origin, such as glial fibrillary acidic protein, during their isolation (Matus et al., 1980), which could account for the presence of α -dystrobrevin-1 and Dp71 in the PSD fractions.

Dystrobrevin Interactions in Mutant Mice

Central to the pathogenesis of muscular dystrophy is the disruption of the DPC in muscle. In dystrophin-deficient muscle, there is a dramatic reduction in the levels of DPC at the sarcolemma, possibly due to a failure of this complex to assemble (Holt and Campbell, 1998). However, to the best of our knowledge, no one has examined the assembly of DPC-like complexes in other tissues. In this paper, we have shown that the dystrobrevin-syntrophin complex in the brain is apparently unaffected by the absence of dystrophin and Dp71 (Fig. 9 A). Furthermore, dystrobrevin-immunoreactivity appears normal in the brains of dystrophin-deficient mice (Blake, D.J., unpublished results). By contrast, in muscle this complex is destabilized in the absence of dystrophin (Fig. 9 B). This difference between the assembly or stability of DPC-like complexes in muscle and brain may explain why the absence of dystrophin or Dp71 in the brain produces a relatively mild phenotype, compared with the devastating muscle pathology seen in patients with DMD and the *mdx* mouse. The analysis of several mouse mutants has elegantly demonstrated that the absence of individual components of the DPC complex in muscle often results in muscular dystrophy (Duclos et al., 1998; Hack et al., 1998; Grady et al., 1999). Therefore, it can be argued that each component of the DPC contributes directly to the pathogenesis of muscular dystrophy. However, our data raises the possibility that the assembly of DPC-like complexes in neurons and glia is largely unaffected by the absence of dystrophin and its isoforms. While these findings seem to preclude a contributory role for β -dystrobrevin in the cognitive dysfunction that affects some DMD patients, it should be emphasized that, due to the refractory nature of the PSD, subtle differences in the assembly of the dystrophin-dystrobrevin complex at postsynaptic sites may go undetected.

In conclusion, we have shown that there are fundamental differences in the composition of DPC-like complexes in neurons and glia, and between the assembly and stability of the DPC in muscle and brain. The identification of β -dystrobrevin as a binding partner for dystrophin and their enrichment in PSDs suggests that both proteins are components of central synapses and thereby define the neuronal DPC. This discovery is particularly relevant because a large proportion of DMD patients have mild cognitive impairment. The identification of β -dystrobrevin in multiple subcellular compartments in the brain could invoke another route for the propagation of extra- or intracellular signals to different regions of the neuron. It is

tempting to speculate that some of the cognitive difficulties experienced by DMD patients may be caused by alterations in the β -dystrobrevin-dystrophin complex at PSDs.

The authors thank Prof. Kay Davies for support and encouragement, Estrella Gonzales and Rosemary Mummery for technical help, and Dr. Nellie Loh for providing fusion proteins and for helpful discussions. We are grateful to Prof. Glenn Morris and Prof. Stan Froehner for supplying the MANDRA1 and SYN1351 antibodies, respectively.

This work was generously supported by the Wellcome Trust (D.J. Blake and P.W. Beesley) and by The Medical Research Council of Canada (R. Hawkes). D.J. Blake holds a Wellcome Trust Career Development Fellowship.

Submitted: 29 July 1999

Revised: 20 September 1999

Accepted: 21 September 1999

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