

Review

Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue

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Abstract. The dystrophin glycoprotein complex (DGC) is a multimeric protein assembly associated with either the X-linked cytoskeletal protein dystrophin or its autosomal homologue utrophin. In striated muscle cells, the DGC links the extracellular matrix to the actin cytoskeleton and mediates three major functions: structural stability of the plasma membrane, ion homeostasis, and transmembrane signaling. Mutations affecting the DGC underlie major forms of congenital muscle dystrophies. The DGC is prominent also in the central and peripheral nervous system and in tissues with a secretory function or which form barriers between functional compartments, such as the

blood-brain barrier, choroid plexus, or kidney. A considerable molecular heterogeneity arises from cell-specific expression of its constituent proteins, notably short C-terminal isoforms of dystrophin. Experimentally, the generation of mice carrying targeted gene deletions affecting the DGC has clarified the interdependence of DGC proteins for assembly of the complex and revealed its importance for brain development and regulation of the ‘milieu intérieur’. Here, we focus on recent studies of the DGC in brain, blood-brain barrier and choroid plexus, retina, and kidney and discuss the role of dystrophin isoforms and utrophin for assembly of the complex in these tissues.

Keywords. Blood-brain barrier, choroid plexus, dystrophin, Dp71, epithelial cell, endothelial cell, homeostasis, kidney, retina, targeted gene deletion, transmembrane signaling, utrophin.

Introduction

The dystrophin glycoprotein complex (DGC) comprises five classes of proteins (dystroglycans, syntrophins, dystrobrevins, sarcoglycans, and sarcospan) made of several members or isoforms, and assembled with either dystrophin or its autosomal homologue utrophin (Fig. 1). The DGC has been studied mainly in the context of muscle dystrophies and cardiomyopathies [1–3]. It is critical for integrity of muscle fibers by linking the actin cytoskeleton to the extracellular matrix (ECM) [4–8]. More recently, its roles as a signaling complex and as a scaffold

for membrane proteins have gained preeminence. Furthermore, the DGC has been recognized to be molecularly heterogeneous and present in numerous tissues, notably in the central and peripheral nervous system, and in tissues with secretory function or forming barriers between functional compartments, such as the blood-brain barrier (BBB), choroid plexus (CP), or kidney. While the functional role of ‘non-muscle’ DGC remains to be clarified in most of these organs, there is compelling evidence for its involvement in brain development, synapse formation and plasticity, as well as water and ion homeostasis. The analysis of mice carrying spontaneous or targeted mutations affecting specific DGC components has clarified the interdependence of DGC proteins for assembly of the complex. These studies have also shown that despite functional redundancy, dystrophin isoforms and

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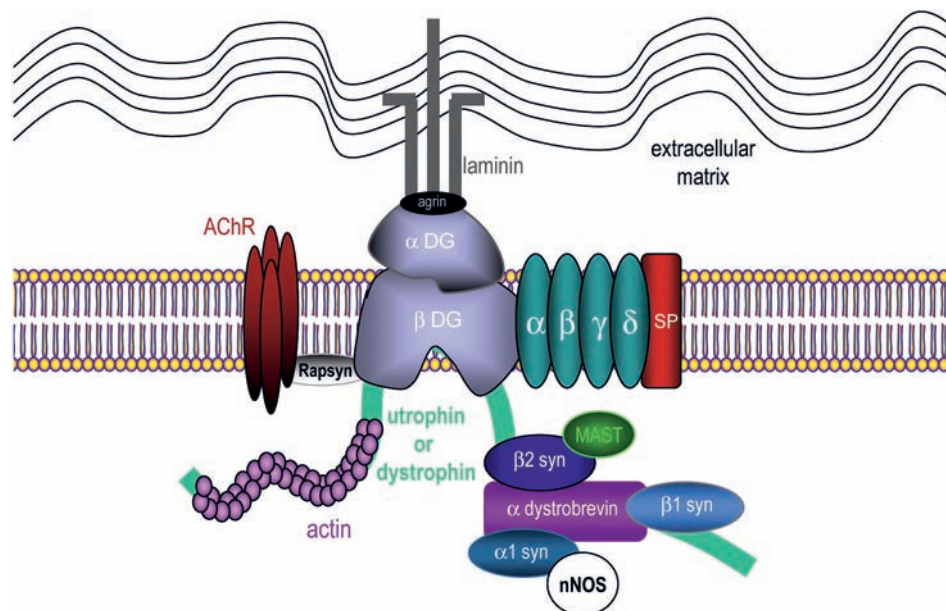


Figure 1. Schematic organization and composition of the DGC at the neuromuscular junction. Dystrophin or utrophin bind to actin filaments via their N terminus. At the C terminus, dystrophin or utrophin are associated with integral and peripheral membrane proteins that can be classified as the dystroglycan complex, the sarcoglycan-sarcospan complex, and the cytoplasmic complex. The cytoplasmic complex includes isoforms of syntrophin (α 1-, β 1-, β 2-syn) and α -dystrobrevin. The sarcoglycan-sarcospan complex comprises isoforms of sarcoglycan (α , β , γ , δ) and sarcospan (SP). The extracellular component of the dystroglycan complex, α -dystroglycan (α -DG), binds to agrin and laminin in the extracellular matrix and the transmembrane isoform β -dystroglycan (β -DG). In turn, β -dystroglycan binds to dystrophin or utrophin, thus completing the link between the actin-based cytoskeleton and the extracellular matrix. Rapsyn is involved in acetylcholine receptor (AChR) clustering. Signaling proteins such as the microtubule-associated serine/threonine kinase (MAST) or the neuronal nitric oxide synthase (nNOS) are recruited to the DGC via PDZ-binding domains.

utrophin are likely to fulfill distinct roles in non-muscle tissue. Here, we will briefly summarize major features of the DGC in skeletal muscle cells and present an overview of recent developments about the DGC in the brain, BBB, retina, and kidney. The major focus of this review is the role of dystrophin isoforms and utrophin for proper assembly and function of the DGC in these tissues.

The DGC in skeletal muscle

The major components of the DGC have been isolated and characterized best in skeletal muscle cells and will be presented briefly (Fig. 1) before discussing their role and localization in 'non-muscle' tissue. The sarcoglycan complex and sarcospan, which are transmembrane proteins linked to the DGC (Fig. 1), will not be considered in this review.

Dystrophin

The dystrophin gene, located on the X chromosome, spans approximately 2.5 Mb and is composed of 79 exons [9, 10]. Duchenne muscular dystrophy (DMD) is caused by mutations causing a frame-shift and abortion of protein translation. Three independently regulated promoters, in muscle, brain, and specifically Purkinje cells in the

cerebellum, control expression of full-length dystrophin [11–17]. In skeletal muscle, dystrophin predominates at the sarcolemma but is also found at the troughs of the postsynaptic membrane along with voltage-gated sodium channels [17, 18]. Several short dystrophin isoforms arise from differential promoter usage. Transcripts from four internal promoters encode proteins of 260, 140, 116, and 71 kDa (Dp260, Dp140, Dp116, and Dp71) [19]. Dp71 is subjected to alternative splicing of exons 71–74 and/or 78, generating at least four Dp71 isoforms [20] with widespread distribution in various non-muscle tissues [21–32]. Full-length dystrophin binds actin near its N terminus and dystroglycan, thereby providing a structural link between the membrane and the cytoskeleton (Fig. 1). Dp71 also carries an actin-binding site [33], suggesting that this short C-terminal isoform fulfills similar functions.

Utrophin

Utrophin was discovered as a cDNA isolated from fetal muscle with high homology to the DMD gene [34]. It is expressed in nearly all tissues, including skeletal muscle, with particularly high levels in lung, kidney, nervous system, and vascular endothelial and smooth muscle cells [25, 31, 32, 35–38], but its function remains largely unknown. Most studies have focused on adult skeletal mus-

cle, where two full-length isoforms of utrophin, differing in their initial few amino acids, have been identified (A- and B-utrophin) [39, 40]. Utrophin is expressed in several structures within skeletal muscle tissue (including blood vessels and nerves) but the dominant utrophin isoform in muscle fibers is A-utrophin. It is confined to the neuromuscular junction (NMJ) and is closely associated with acetylcholine receptors (AChRs) at the crests of the postsynaptic membrane [17, 18, 41–44]. In the present review, no further distinction will be made between the two full-length utrophin isoforms, which are simply referred to as utrophin.

Dystroglycan

Dystroglycan was the first member of the DGC to be cloned [45]. The dystroglycan gene, *DAG1*, comprises two exons and encodes a single polypeptide that is post-translationally cleaved to yield two glycoproteins [46, 47]. Dystroglycan, which anchors the DGC to the sarcolemma and interacts with multiple components of the ECM, is a central component of the complex. Its function critically depends on multiple glycosylation sites which are regulated in a tissue-specific manner [48]. Full chemical deglycosylation of dystroglycan results in loss of ligand-binding activity [5], and abnormal glycosylation is associated with several congenital muscular dystrophies and abnormal central nervous system (CNS) development [2, 49–51]. α -Dystroglycan (α -DG) is located extracellularly where it functions as a laminin/agrin receptor involved in basal membrane formation and synaptogenesis [52–56]. β -Dystroglycan (β -DG) spans the membrane and binds either dystrophin or utrophin at a WW domain near its C terminus. Caveolin 3 binds to the same domain [57], suggesting a potential competitive interaction modulating the membrane anchoring of the DGC [58]. β -DG also binds rapsyn, a protein of the NMJ that is required for clustering of AChRs [59, 60], as well as signaling molecules, such as Grb2, a growth factor receptor-bound adapter protein [61, 62]. The concept that β -DG acts as a scaffolding protein has recently been strengthened by the demonstration that it binds to both MEK and ERK, thereby modulating the ERK-MAP signaling cascade [63].

However, the significance of dystroglycan extends far beyond striated muscle, as it is the most broadly expressed DGC component in both developing and adult tissues [2, 64–66], typically in cell types apposed to a basal lamina [67]. Furthermore, dystroglycan plays an important role in the peripheral nervous system, regulating Schwann cell function and the organization of nodes of Ranvier [68, 69]. Finally, the binding of α -DG to neurexins, neuron-specific cell surface proteins, suggests a novel role for dystroglycan as an intercellular cell adhesion molecule in neurons [70].

Syntrophin

The syntrophin (syn) family is composed of five members, $\alpha 1$ -, $\beta 1$ -, $\beta 2$ -, $\gamma 1$ -, and $\gamma 2$ -syn encoded by different genes. $\alpha 1$, $\beta 1$, $\beta 2$, and $\gamma 2$ are expressed in skeletal muscle [71–73]. $\beta 2$ -syn is restricted to the NMJ, whereas $\alpha 1$ -, $\beta 1$ -, and $\gamma 2$ -syn are also localized along the sarcolemma [73, 74]. Accordingly, $\alpha 1$ - and $\beta 1$ -syn are associated with dystrophin and $\beta 2$ -syn with utrophin [75]. Syntrophins are also expressed in other tissues, such as brain [26, 76–79], retina [80, 81], kidney [32, 82, 83], and liver [84–86]. The γ -syntrophins are most abundant in the brain with $\gamma 1$ -syn being neuron specific [73].

Syntrophins carry a PDZ-binding domain interacting with a variety of signaling molecules and membrane proteins (Fig. 1), including neuronal nitric oxide synthase (nNOS) [87], aquaporin 4 (AQP4) [88], inwardly rectifying K^+ channels [89], muscle voltage-gated sodium channels [77, 90], and stress-activated protein kinase 3 [91]. In addition, $\beta 2$ -syn recruits the serine/threonine kinases MAST and SAST to the DGC [92]. The multiple protein-protein interactions mediated by syntrophins underscore the role of the DGC as a scaffold regulating surface expression of channel proteins and subcellular localization of signaling complexes.

Dystrobrevin

Dystrobrevin is a member of the dystrophin-related protein family with significant homology to the C terminus of dystrophin [93, 94]. Two isoforms, α - and β -dystrobrevin (α - and β -DB), are encoded by different genes [95–97]. The α -DB gene gives rise to at least five splice variants: α -DB1, -2, and -3 are present at the sarcolemma; α -DB1 is restricted to the NMJ whereas α -DB2 has a distribution similar to that of dystrophin [97–99]. Both isoforms bind directly to dystrophin and utrophin through reciprocal coiled-coil regions in each protein [100, 101]. β -DB is absent from striated muscle but is expressed in many non-muscle tissues where it associates with Dp71 and utrophin [26, 29]. A potential signaling molecule containing two MAGe homology domains has recently been identified which selectively binds α -DB and is colocalized with the DGC in brain, muscle, and peripheral nerves [102].

Mutant mice models for studying assembly and function of the DGC

Although no human mutations have been found in genes encoding dystroglycan, syntrophin or dystrobrevin [2, 50], mouse lines carrying targeted gene deletions for these DGC proteins have been generated to study their function. These mutants are presented here in the context of the role and assembly of the DGC in striated muscle and will be discussed in more detail further on.

Dystrophin-null mice

mdx mice [103] lack full-length dystrophin due to a spontaneous point mutation in exon 23 of the DMD gene [104]. These mice exhibit moderate signs of skeletal muscle dystrophy but show little weakness and have a near normal lifespan. This mild phenotype is due in part to compensatory up-regulation of utrophin [105, 106]. However, in the absence of dystrophin, α -DB1 and -2, as well as α 1-, β 1-, and β 2-syn disappear from the sarcolemma but remain at the NMJ [106], where they probably associate with utrophin [75]. The sarcolemmal loss of α 1-syn also affects nNOS and AQP4, which are not properly targeted to the membrane in *mdx* mice [107, 108]. Human studies have reported similar findings, showing that α 1-syn, α -DB1, and α -DB2 were reduced in muscle from DMD patients [109, 110]. Despite the mild phenotype of *mdx* mice, a typical diagnostic criterion for muscular dystrophies remains: creatine kinase plasma levels are elevated in *mdx* mice [103].

In *mdx* mice, short C-terminal isoforms of dystrophin, such as Dp71, are not affected. Mice lacking all dystrophin isoforms (*mdx*^{3Cv}) have been generated to overcome this limitation [111, 112]. However, the presence of dystrophin-expressing “revertant” muscle fibers has been reported in both *mdx* and *mdx*^{3Cv} mice [16], probably due to exon-skipping events occurring during all stages of development. *DMDmdx*- β geo mice carry a transgene inserted 3' to exon 63 of the dystrophin gene, affecting translation of all dystrophin isoforms, including Dp71 [113]. These mice develop a dilated esophagus and also cardiac hypertrophy. Both *mdx*^{3Cv} and *DMDmdx*- β geo mice display essentially the same muscle pathology as *mdx* mice but have additional defects reflecting the loss of DGC in non-muscle tissue and resulting in a shorter lifespan.

Finally, mutant mice carrying targeted deletions of the DMD gene have been generated [114]. DMD-null males are sterile and exhibit severe degeneration and regeneration of myofibers in striated muscle, as seen in DMD patients [114]. In contrast, mutants with a brain-specific inactivation of full-length dystrophin exhibited no histological abnormality in striated muscle nor in various non-muscle tissues and were indistinguishable from their wild-type littermates [114]. However, a detailed behavioral evaluation of these mice will be necessary to confirm a lack of neurological phenotype.

Utrophin-null mice

Utrophin^{0/0} mice have no morphological defects, breed normally, and have a normal lifespan [115]. Dystrophin expression remains unchanged at the sarcolemma but is up-regulated at the NMJ [116]. In contrast, β -DG and dystrobrevin are normally distributed in utrophin-deficient skeletal muscle. Morphologically, the NMJ of utrophin^{0/0}

mice is normal at birth but fewer postsynaptic folds develop thereafter, along with a modest decrease in AChR density [116, 117]. Therefore, utrophin is dispensable for clustering of AChRs at the NMJ, although utrophin is lost from the NMJ in the absence of AChRs [118, 119]. In conclusion, utrophin appears to be dispensable for assembly of a DGC but contributes to proper maturation of the postsynaptic apparatus [60, 117].

Utrophin-dystrophin double-knockout mice

The functional redundancy between utrophin and dystrophin has been confirmed in utrophin^{0/0}/*mdx* double-mutant mice, which show major symptoms of DMD, including severe muscle weakness, pronounced growth retardation as well as reduced lifespan [120]. In addition, β -DG is down-regulated, whereas dystrobrevin and β 2-syn are undetectable at the NMJ. Nonetheless, laminin- β 2, agrin, and rapsyn are unaffected at synapses of utrophin^{0/0}/*mdx* mice, indicating that postsynaptic differentiation can occur not only in the absence of both utrophin and dystrophin but also when the DGC is largely disrupted [120]. However, a compensation by dystrobrevin or dystrophin-related protein 2 [121] has not been excluded in these mice.

Syntrophin- and dystrobrevin-null mutations

In α 1-syn^{0/0} mice, β 1- and β 2-syn are up-regulated whereas utrophin is lost from the NMJ, suggesting a mandatory association [75, 122]. In addition, AChRs and ACh esterase are significantly decreased, whereas nNOS is absent from the postsynaptic membrane and the sarcolemma [122]. Mice lacking β 2-syn have no apparent muscle phenotype, except for elevated AChR number at the NMJ [123]. That the absence of this protein, which binds to several signaling molecules, does not cause a more severe phenotype is surprising. Even in mice lacking both of these syntrophin isoforms there is no evidence of muscle dystrophy although they run significantly less on voluntary exercise wheels than wild-type mice of either parent strain [123]. In the absence of damage to muscle fibers, this deficit may be due to an unrelated defect, perhaps affecting metabolism or neuronal function. Analysis of the NMJ of α 1/ β 2-syn-null mice has revealed structural defects similar in nature but more severe than those observed in the α 1-syn^{0/0} mice. These alterations occurred despite the presence of normal levels of dystrophin, dystrobrevin, and sodium channels [123]. Altogether, these observations point to extensive functional redundancy between α 1- and β 2-syn.

Analysis of α -DB^{0/0} mice has revealed a dual role for α -DB in the pathogenesis of muscle dystrophy and in AChR stabilization at the NMJ [124, 125]. These mice develop a mild form of dystrophy without disruption of the DGC

at the sarcolemma, indicating that dystrophy might also develop as a result of impaired DGC-dependent signaling. α -DB may be part of a scaffolding or signaling complex required to assemble components of the postsynaptic membrane during synapse formation [126]. Finally, mice lacking β -DB do not suffer from dystrophy since this isoform is not expressed in skeletal muscle. Nevertheless, the DGC is altered at the membrane of cortical renal tubules and hepatic sinusoids [82], underscoring the importance of β -DB in non-muscle tissues.

Mutations affecting dystroglycan

Dystroglycan is essential for embryonic development, as reflected by disruption of the Reichert's membrane surrounding the embryo, resulting in early lethality of null mutant mice [65]. *In vitro*, embryonic stem cells of *DAG1*-null mice form embryoid bodies with a disrupted basement membrane. However, when allowed to differentiate further, these cells can give rise to skeletal muscle, cardiac muscle, and neurons [127]. Furthermore, chimeric mice are rescued from early embryonic lethality and exhibit normal striated muscle differentiation [127]. These mice have severely reduced levels of utrophin and AChRs at the NMJ, confirming the critical role of dystroglycan in the formation of the NMJ [47].

Importantly, dystroglycan function is severely impaired by glycosylation defects. Mutations in at least six genes encoding glycosylation enzymes are associated with congenital muscular dystrophies or myopathies [51, 128–132] commonly termed dystroglycanopathies [50]. The mutations are associated with hypoglycosylation of α -DG and concomitant loss of binding to laminin, agrin, neuroligin, or perlecan [133–137]. For instance, the *Large* gene, which encodes a putative bifunctional glycosyltransferase, is mutated in the myodystrophy (*myd*) mouse [133] and in congenital muscular dystrophy type 1D (MDC1D) [138]. *myd* mice exhibit deficits in neuronal migration (lissencephaly), runting, an abnormal gait, cardiomyopathy, and have a shortened lifespan. Surprisingly, dystrophin and other DGC members are still present at the sarcolemma of muscle cells devoid of dystroglycan [139, 140], suggesting alternative mechanisms for membrane anchoring of the DGC [50]. The significance of dystroglycanopathies in the brain will be discussed below.

Altogether, the results summarized in this section show that muscle dystrophies are associated with loss-of-function mutations independently affecting multiple members of the DGC. The phenotype of most animal models is not identical to that of patients with congenital muscle dystrophies, probably due to the complex, tissue-specific roles assumed by the DGC. These results also show that the function and assembly mechanisms of the DGC in skeletal muscle cells cannot be generalized to non-mus-

cle tissues because most functions depend on partner proteins with a cell-specific distribution and regulation.

The DGC in non-muscle tissues

In striated muscle cells, three major functional domains can be distinguished in the DGC: (i) the actin-binding domain of utrophin or dystrophin which links the complex to the cytoskeleton; (ii) the transmembrane protein β -DG and its peripheral isoform α -DG which anchor the DGC to the cell membrane and provide contact with the ECM; (iii) the binding sites to signaling proteins located on β -DG, syntrophin, and dystrobrevin. In non-muscle tissues, the DGC typically contains fewer components associated with a short C-terminal isoform of dystrophin, such as Dp71 or with utrophin, although the failure to detect some DGC members may be due to technical limitations. Nevertheless, the DGC is usually concentrated at membranes facing a basal lamina, suggesting an important signaling function, and its components carry binding sites for membrane channels or transporters. Below, we review recent developments in the molecular composition, localization, and functional role of the DGC in four major organs and tissues: brain, BBB and CP, retina, and kidney, in which the DGC has been characterized extensively in mutant mice.

Brain

Studies of the DGC in brain have addressed mainly its role in mediating brain abnormalities and mental retardation affecting numerous patients with congenital muscle dystrophies, as well as its role in synapse formation and plasticity [141–146]. Members of the DGC are present in specific neurons, astrocytes, and radial glia [19, 23, 26, 30, 35, 37, 64, 145, 147–155], usually associated with either dystrophin isoforms or with utrophin. Thus, full-length dystrophin is neuron specific and is present in the hippocampus, cerebral cortex, and cerebellum, associated with dystroglycan, as well as dystrobrevin and syntrophins [26, 76, 151, 156, 157]. Dp140 is present selectively in the CNS and kidney and is particularly highly expressed prenatally; it is distributed primarily in astrocytic processes, outlining blood vessels and in the meninges [158]. Dp71 is present in dentate gyrus granule cells and in the olfactory bulb [23, 159] and is predominantly found in astrocytes (see below). Utrophin has a distribution in neurons largely complementary to dystrophin, being highly abundant in brainstem [37]. Moreover, utrophin is highly expressed in brain microvessel endothelial cells (see below).

Dystroglycan has a widespread distribution in the brain, including regions lacking dystrophin and utrophin [64, 155]. Besides its distribution in neurons with other DGC

members, dystroglycan is highly abundant in astrocytes, such as the Bergman glia [151, 160] and glial limitans [49, 135]. In neurons, the DGC is localized postsynaptically at inhibitory synapses, colocalizing with GABA_A receptors [153, 157, 158]. However, other studies have shown that dystrophin, β -DB, and syntrophins are enriched in preparations of postsynaptic densities (PSDs) [147, 161, 162], suggesting that both inhibitory and excitatory synapses might contain a DGC. The functional significance of the DGC in PSDs is further underscored by the binding of nNOS to α 1-syn [78, 87]. In turn, nNOS binds to PSD-93 and PSD-95, which are involved in N-methyl D-aspartate (NMDA) receptor clustering [87]. Therefore, the DGC might recruit nNOS in excitatory synapses, where it is activated by NMDA-receptor-mediated Ca²⁺ influx. This hypothesis is further supported by the demonstration that nNOS mRNA expression depends on dystrophin in cultured human neurons [163].

To understand whether alterations in the DGC in neurons underlie cognitive impairments in DMD patients, *mdx* mice have been extensively studied for defects of neuronal function. At the morphological level, the absence of full-length dystrophin is accompanied by impaired synaptic clustering of GABA_A receptors [153], suggesting a role for dystrophin in regulating GABAergic transmission in a subset of inhibitory synapses. Thus, this deficit has been associated with a reduction in inhibitory transmission in Purkinje cells [164] and with altered short- and long-term synaptic plasticity in CA1 pyramidal cells [165, 166]. While some studies have observed no impairment in spatial learning or long-term potentiation in the absence of dystrophin [167, 168], a reassessment of *mdx* mice has revealed altered long-term retention, but not acquisition in both spatial and non-spatial learning tasks [166]. *mdx*^{3Cv} mice show enhanced anxiety-related behaviors and reduced locomotion but are otherwise no more impaired than *mdx* mice in learning and memory tasks [169], which is in line with the predominant expression of full-length dystrophin in hippocampal pyramidal cells.

Pathological studies of brains of DMD patients with cognitive impairments have shown reductions in brain weight, preferential loss of neuronal populations that normally express dystrophin, and small cortical ischemic infarcts [141], suggesting that the absence of dystrophin increases neuronal susceptibility against hypoxia-induced injury. In line with this hypothesis, CA1 pyramidal neurons in a hippocampal slice preparation of *mdx* mice have been found to be more vulnerable to hypoxia [170] and could be protected by pretreatment with diphenylhydantoin, an anticonvulsant that blocks both sodium-dependent action potentials and low-threshold transient calcium channels. This increased neuronal vulnerability might contribute to the development of cognitive deficits in DMD patients [170].

The role of utrophin in brain is not known. Interestingly, unlike in muscle cells, utrophin is not aggregated at postsynaptic sites but is localized along the membrane of neuronal somata and proximal dendrites [37], suggesting that its function is unrelated to synaptic transmission. A potential neuroprotective role of utrophin has been uncovered in a mouse model of temporal lobe epilepsy induced upon unilateral injection of kainic acid into the dorsal hippocampus of adult mice [171], and in which extensive dispersion and hypertrophy of granule cells occur in the dentate gyrus. These changes are accompanied by a prominent overexpression of utrophin in granule cells [159]. Utrophin^{0/0} mice exhibit an increased sensitivity to kainate-induced excitotoxicity, as shown by increased mortality and faster progression of the lesion [172] and a significant reduction in the number of hypertrophic granule cells, suggesting that utrophin contributes to protect these neurons against pathological insults, in particular stimuli leading to cellular hypertrophy [172].

By far the major functional contribution of the DGC in brain is assumed by dystroglycan, as revealed by the profound brain malformations occurring in muscle-eye-brain disease and in Fukuyama congenital muscular dystrophy [173, 174], two congenital muscle dystrophies associated with α -DG loss of function due to mutations in glycosylation enzymes [135]. Similar brain malformations could be reproduced experimentally in *myd* mice [135] and in mutant mice with a brain-specific deletion of *DAG1* under the control of an astrocyte-specific promoter [49], demonstrating that dystroglycan requires glycosylation for proper function and is essential for regulating cell migration and differentiation during development, as well as synaptic plasticity in adult brain. In the brain of *myd* mice, other members of the DGC, including dystrophin, were not targeted appropriately to postsynaptic sites and to glial endfeet [135], underscoring the role of dystroglycan-mediated function for this process, unlike in striated muscle cells. Despite this deficit, dystroglycan-deficient neurons in culture form synapses containing GABA_A receptors and gephyrin clusters opposite GABAergic terminals [158], indicating that the DGC is not required for the development of these synapses.

Altogether, these findings reveal that the DGC expressed in cells of the astrocytic lineage plays an essential role during brain development, whereas the neuronal DGC, which is localized selectively in specific subsets of synapses in adult brain, most likely modulates synaptic function and plasticity, and might be neuroprotective against ischemic damage and other stimuli leading to hypertrophy. This conclusion is in line with reports that full-length dystrophin expression becomes detectable during the third postnatal week in rodents [37], after neuronal migration and synaptogenesis are largely completed. The reason why the DGC is restricted to specific neuronal populations in the brain is open to speculation.

BBB and CP

Endothelial cells of brain microvessels form the BBB, thereby contributing to the protection of the brain against variations in the chemical composition of the blood [175]. Astrocytes contribute to the formation of the BBB during development by inducing tight junctions between endothelial cells [176]. Brain vessels, including capillaries, are a prominent site of expression of DGC proteins, expressed in both endothelial cells and astrocytic endfeet [26, 37, 79, 177–179]. In addition, DGC proteins are also present in the CP [31], suggesting a potential role in water homeostasis and regulation of transport mechanisms across the BBB, as well as cerebrospinal fluid production.

Utrophin is abundantly expressed along with $\beta 2$ -syn in brain endothelial cells [31, 37] but not in astrocytic endfeet (Fig. 2) [31]. In these studies, no other DGC member protein, including Dp71 and dystroglycan, has been detected in endothelial cells of brain capillaries, unlike previous reports describing the presence of dystroglycan in brain blood vessels [96, 155]. The discrepancy between these results might reflect a possible heterogeneity between capillaries and arterioles. In any case, the molecular composition of the DGC in endothelial cells appears to be simpler than in striated muscle or in neurons.

No $\beta 2$ -syn immunoreactivity is detectable in brain blood vessels from utrophin^{0/0} mice, demonstrating a direct association of these proteins in endothelial cells [31], as in striated muscle. However, the loss of utrophin and $\beta 2$ -syn did not affect the localization of the MRP2-type of ABC transporter or the glucose transporter 1 (GLUT1), which are present in the luminal membrane of endothelial cells in the brain [31]. So far, we do not know whether specific signaling proteins or transporters are associated with the DGC in endothelial cells. No alteration in staining for utrophin and $\beta 2$ -syn could be observed in brain blood vessels of *mdx*^{3Cv} mice [31], in agreement with the absence of detectable Dp71 in endothelial cells of wild-type mice (Fig. 2). However, the absence of Dp71 from astrocytic endfeet (see below) during development has been suggested to affect the development of the BBB, leading to an altered expression of the tight junction marker zonula occludens 1 (ZO-1) in old *mdx* mice [180]. These authors therefore speculate that altered cross-talk between glial endfeet and endothelial cells in the absence of dystrophin might contribute to the neurological dysfunctions associated with DMD [181].

Astrocytic endfeet surrounding brain blood vessels exhibit prominent Dp71 expression (Fig. 2) along with β -DG, syntrophin isoforms, and α -DB1 [26, 31, 182–184]. High-resolution immunoelectron microscopy studies have demonstrated that AQP4 and the inwardly rectifying K⁺ channel, Kir4.1, are localized selectively in the astrocytic membrane that is in direct contact with the basal lamina

facing the blood vessel [179, 185–187]. Both proteins bind to the PDZ domain of $\alpha 1$ -syn [185], suggesting that the DGC anchors these proteins at the membrane. The importance of this association has been demonstrated in α -syn^{0/0} mice, in which the membrane localization of AQP4 and Kir4.1 is disrupted, causing a delay in clearance of extracellular K⁺ after neuronal activation and an increase in seizure susceptibility [178, 185, 188, 189]. In *mdx*^{3Cv} mice, no DGC proteins can be detected at perivascular endfeet of astrocytes [31], suggesting a complete disruption of the DGC affecting also the localization of AQP4 [190]. However, earlier studies reported that syntrophin, dystrobrevin, and dystroglycan were not altered in these mice [26, 112]. While these discrepancies might reflect incomplete penetrance of the mutation, expression of DGC proteins, as detected by Western blotting, may remain unaltered in mutant mice even when their localization is disrupted.

In CP epithelium, a DGC distinct from those found in brain microvessels has been described [31, 37, 191]. It is formed by utrophin along with $\beta 1$ - and $\beta 2$ -syn and β -DB [31]. Unexpectedly, β -DG was not detected in CP epithelial cells, whereas α -DG was targeted apically [31] despite the presence of a basal lamina between the epithelial cells and the underlying endothelium. Therefore, it remains unclear how the DGC is anchored at the epithelial cell membrane and whether it is linked to the ECM. In CP of utrophin^{0/0} mice, $\beta 1$ - and $\beta 2$ -syn were undetectable whereas β -DB was mislocalized to an intracellular compartment, suggesting that these proteins are differentially dependent on utrophin for proper membrane targeting [31]. These alterations had no apparent consequences for the morphology of epithelial cells, although no compensation by full-length dystrophin or Dp71, which are normally not detectable in the CP, could be observed in utrophin^{0/0} mice [31].

Taken together, these observations suggest the presence of at least three distinct DGCs in the BBB and CP. The analysis of mutant mice shows a clear dependence of DGC proteins for the presence of either Dp71 or utrophin for proper assembly of the complex, and provides no evidence for compensatory up-regulation of another member of the DGC. While the function of the DGC at the BBB and in the CP remains to be uncovered, its role as a scaffold for membrane anchoring of AQP4 and Kir4.1 channels is well established in astrocytic endfeet.

Retina

In analogy to the brain, multiple molecularly distinct DGCs are found in the retina, notably in photoreceptors, neurons, Muller glial cells (MGCs), and blood vessels [192–194]. The predominant dystrophin isoforms are full-length dystrophin, Dp260, Dp140, and Dp71; utrophin is also present, mainly at the same sites as Dp71 [195–200].

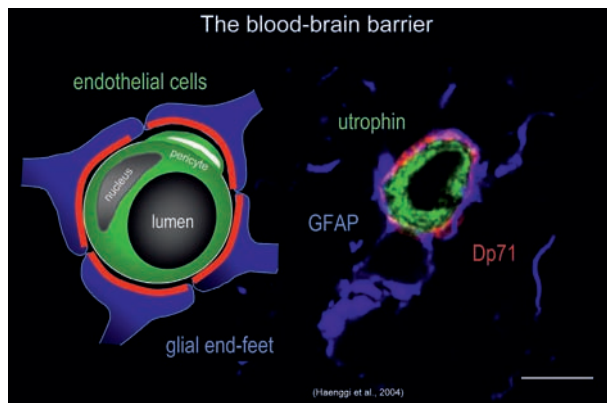


Figure 2. Segregated distribution of utrophin (green) and Dp71 (red) at the BBB and in astrocytic endfeet (GFAP-staining, blue). A schematic representation of a blood vessel surrounded by endfeet is depicted on the left. Utrophin immunoreactivity is localized in endothelial cells, whereas Dp71 overlaps with the GFAP signal in glial endfeet forming a ring around the blood vessel. Scale bar, 5 μ m.

In the outer plexiform layer (OPL), where photoreceptors form synapses with horizontal and bipolar cells, the DGC contains dystrophin, dystroglycan, α 1-syn, and α -DB [80, 199, 201]. The organization of the DGC differs from that of other neurons, being localized presynaptically in photoreceptor terminals [197, 198, 202–207]. Possible species differences exist, with both a pre- and a postsynaptic DGC being present in the OPL of rabbit and porcine retina [81, 207]. A DGC of similar composition is found also at the IPL (synapses between bipolar/amacrine cells and ganglion cells), although α -DG was not detectable

[199]. In contrast, an atypical DGC is found at the outer segment (OS) of photoreceptors, containing β -DG and α 1-syn but lacking either dystrophin or utrophin [199]. A major DGC is present in the inner retina where MGC endfeet join to form the inner limiting membrane (ILM), separating the retina from the vitreous body. Across this barrier, K^+ ions are released into the vitreal space through Kir4.1 channels, which are concentrated at the MGC endfeet [208], contributing to K^+ buffering [209]. In addition, AQP4 is localized at the perivascular membrane of MGC endfeet [210] to control retinal water transport [211]. Several members of the DGC have been detected in MGCs (β -DG, α 1-, β 1-syn, α -DB) and their endfeet (Dp71, utrophin, dystroglycan) [28, 199–201, 208, 212]. The complex associates with Kir4.1 and AQP4, similar to the DGC found in perivascular astrocytic endfeet in brain, and most likely fulfills a similar functional role [213].

The segregation of various DGCs to different retinal layers has allowed detailed analyses of the role of dystrophin isoforms for their assembly and subcellular localization. For example, in retina of *mdx*^{3Cv} mice, β -DG protein levels are reduced in the ILM and OPL [199], whereas α 1-syn and α -DB are not affected [199]. Furthermore, clustering of Kir4.1 is disrupted in MGC endfeet of *mdx*^{3Cv} [208]; unexpectedly, it is not affected in α 1-syn^{0/0} mice [212], pointing to a differential mechanism compared with brain blood vessels. Partial disruption of the DGC has also been reported in the retina of Dp71-null mice, which exhibit reduced levels of β -DG at the ILM, whereas α 1-syn is

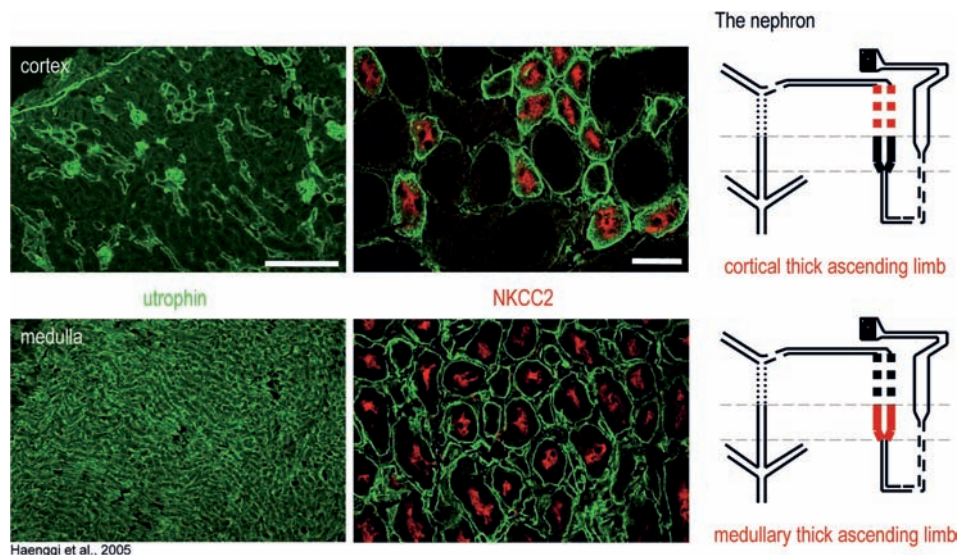


Figure 3. Differential expression and cellular distribution of utrophin in mouse kidney. A schematic drawing of the nephron is depicted on the right panel. The cortical and medullary thick ascending limbs are colored in red. Low-resolution immunofluorescence images (left panel) depict utrophin (green) distribution in the cortex and medulla. In the cortex, utrophin staining is heterogeneous, labeling glomeruli and few cortical segments. Using a renal segment-specific marker, the $Na^+/K^+/Cl^-$ cotransporter 2 (NKCC2) (red, right panel), which is polarized along the apical membrane of cortical and medullary thick ascending limbs, we could identify a utrophin-positive segments. In the medulla, utrophin exhibits a homogenous distribution and double staining with NKCC2 revealed medullary thick ascending limbs as utrophin-positive segment (lower middle panel). More renal markers have been investigated [32]. Scale bars, 200 μ m (utrophin), 30 μ m (NKCC2).

not affected [200]. Unlike in *mdx*^{3Cv} mice, β -DG expression is normal at the OPL, possibly due to association with Dp260 [200]. Therefore, Dp71 is important for the assembly of the DGC selectively in MGC endfeet. This conclusion is supported by the fact that β -DG is disrupted at the OPL of mice lacking full-length dystrophin and Dp260 [214, 215].

The retina also provides a functional read-out applicable for both mutant mice and DMD patients. Indeed, electroretinogram (ERG) anomalies are among the best-characterized non-muscular manifestations of DMD. Analysis of the dark-adapted ERG has revealed a reduction in the amplitude of the b-wave response in 80% of DMD patients [216–219]. A prolonged implicit time of the b-wave has been observed in ERG of mice lacking full-length dystrophin and Dp260 [215], whereas Dp71-null mice showed no significant change [200]. By comparing ERG alterations in patients with distinct mutations, Pillers et al. [220] suggested that in addition to Dp260, other C-terminal isoforms contribute to the generation of the b-wave. This hypothesis was confirmed by the demonstration that *mdx*^{3Cv} have b-waves with reduced amplitude and increased implicit time [221].

As expected from studies in the brain, *myd* mice exhibit major morphological alterations in the retina, affecting MGCs as well as neurons. A similar phenotype was seen, in addition, in a novel mutant mouse line, *Large*^{vls} mice [134, 222]. These mice carry a mutation in a new allele of *Large*, named veils (vls), and share phenotypic characteristics with the *myd* mutation [222]. These findings confirm that defective dystroglycan glycosylation contributes to retinal abnormalities.

Kidney

Epithelial cells in the nephron express numerous DGC proteins, forming several distinct DGCs. As the nephron is organized in distinct segments to sequentially reabsorb ions and solutes from the glomerular ultrafiltrate, it represents an attractive organ to study the localization and specific distribution of DGC proteins [29, 32, 66, 223–225]. Dystroglycan is expressed early by epithelial cells in the developing kidney, whereas in adult tissue only low levels are detectable [66, 67], suggesting that dystroglycan is more important for morphogenesis of renal epithelial cells than during the adult stage. Similarly to dystroglycan, Dp140 is only expressed during kidney development [226]. Low levels of Dp71 are detectable in adult kidney [32, 82, 224, 226–230], although a specific splice variant has been reported to be abundant, in particular in the cortex [29]. In contrast, utrophin is prominent in all segments of the nephron except proximal tubules (Fig. 3) [32]. On the subcellular level, utrophin is specifically localized along the basal, but not lateral, membrane of tubular epithelial cells, demonstrat-

ing that it is restricted to sites of contact with the basal lamina (Fig. 3). In contrast, the DGC is polarized along the basolateral membrane of cultured kidney epithelial cells [231], suggesting altered targeting *in vitro* in the absence of the basal lamina. Using specific markers to identify distinct segments of the nephron, utrophin has been shown to be associated with different members of the DGC in a segment-specific manner (Fig. 3). In particular, α 1- and β 1-syn have a restricted distribution, whereas β 2-syn and β -DB are ubiquitous [29, 32]. These findings indicate that Dp71 isoforms and utrophin are the major partners of the DGC in the nephron and that the functional specialization of the tubule is reflected in the segment-specific distribution of certain DGC proteins.

Possible alterations in DGC assembly and targeting have been investigated in *mdx*^{3Cv} mice to test the role of Dp71. β 2-syn staining was altered in cortical renal tubules, Bowman's capsule and glomeruli, whereas the localization of β -DB, α -DB-1, utrophin, α 1-syn and β 1-syn was not affected [29]. These findings suggest differential dependence of β 2-syn and other DGC proteins on Dp71 for complex formation. In a complementary study using utrophin^{0/0} mice, we have demonstrated that β 2-syn localization is not impaired in cortical segments in the absence of utrophin, whereas it is lost in all segments expressing high utrophin levels in wild-type mice [32]. Again, other DGC proteins were either not affected in mutant mice (β 1- and α 1-syn) or were upregulated (Dp71, β -DG, and dystrobrevin) (Fig. 4), indicating that compensatory mechanisms are activated to preserve most of the DGC in either *mdx*^{3Cv} or utrophin^{0/0} mice [32].

To directly demonstrate this compensatory up-regulation, utrophin-deficient mice were cross-bred with *mdx*^{3Cv} mice to generate utrophin^{0/0}/*mdx*^{3Cv} double mutants. These mice have a reduced lifespan [120], and only few reach the adult stage. Nevertheless, analysis of the nephron has revealed a complete disruption of the DGC, highlighting the functional redundancy between utrophin and dystrophin in cells coexpressing both proteins [32].

The complex, segment-specific molecular organization of the DGC in the nephron suggests multiple functional roles related to ion transport mechanisms. In analogy to skeletal muscle cells, where the DGC provides membrane stability during muscle contractions [4], renal epithelial cells might also have a DGC to resist the high osmotic pressure of the hypertonic interstitial fluid surrounding medullary tubules [232]. The high abundance of utrophin in these segments of the nephron, unlike in the renal cortex, supports this idea. Although Dp71 is upregulated in utrophin^{0/0} mice, the compensation is only partial because β 2-syn is lost from the DGC. The reduced life expectancy of double-mutant mice where no compensation is possible in the kidney might be due to renal dysfunction. However, this hypothesis remains to be tested.

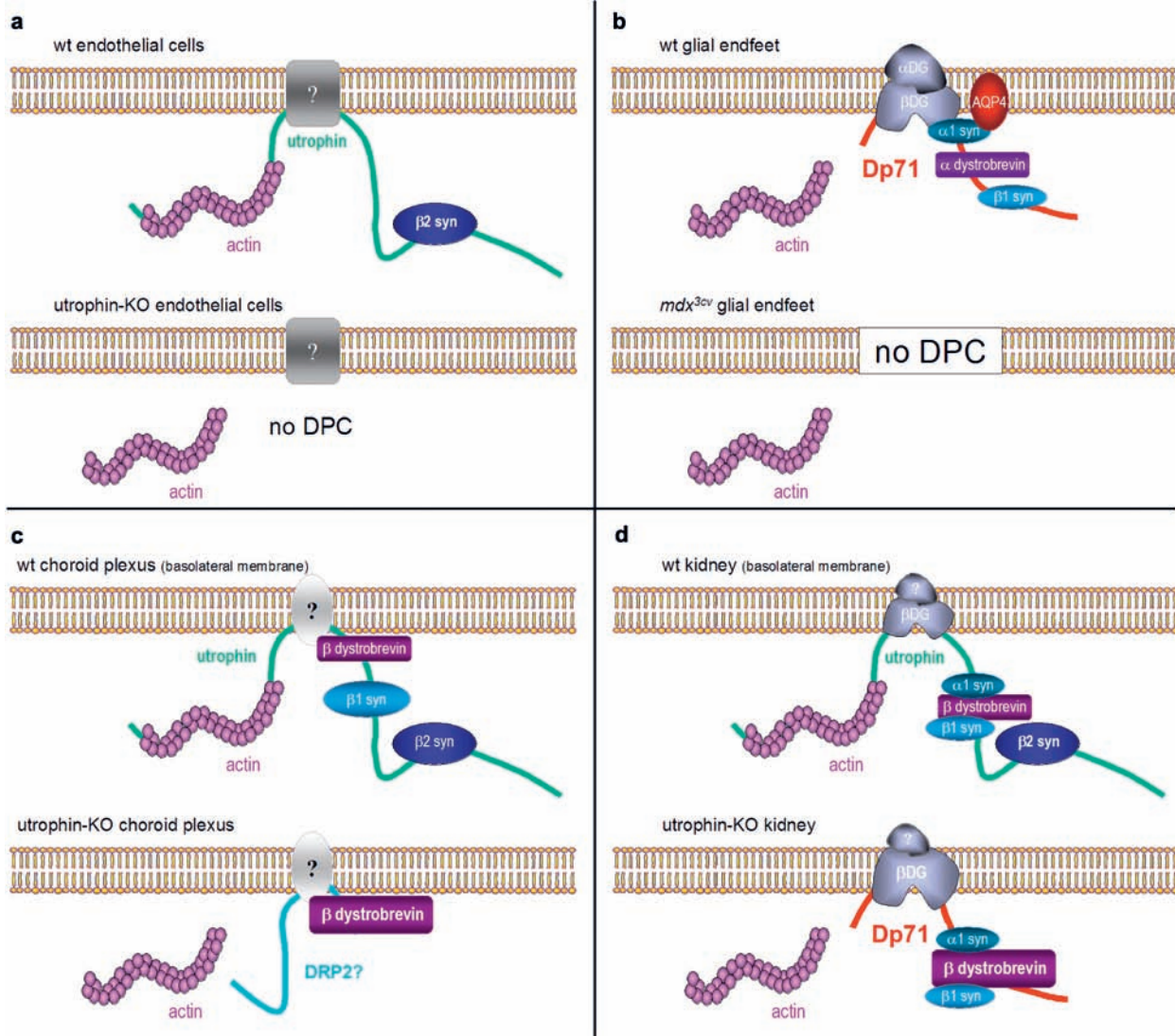


Figure 4. Molecular composition of the DGC in four tissues of wild-type and either utrophin^{0/0} or mdx^{3Cv} mice. Note the mandatory association of β₂-syn with utrophin in kidney, CP, and endothelial cells. In utrophin^{0/0} kidney, the DPC is partially rescued by compensatory upregulation of Dp71, but it is unclear whether it binds the actin cytoskeleton. In blood vessels (endothelial and glial endfeet), no compensation occurs and the DPC is disrupted, along with AQP4. An intermediate situation occurs in the CP, where β-DB is partially retained, possibly due to upregulation of dystrophin-related protein 2 (DRP2).

Finally, for proper function of renal epithelial cells, ion channels, exchangers, and transporters must be targeted to either the apical or basolateral membrane [233]. The DGC may be involved in anchoring renal transporters and channels to the basal membrane. So far, however, no protein has been identified for which the DGC serves as a scaffolding protein, similarly to AQP4 and Kir4.1 in astrocytes.

Conclusions

The characterization of the DGC in non-muscle tissues has revealed an unexpected heterogeneity in molecular composition, in particular with respect to the presence

of α- or β-DG. The mechanism of membrane anchoring and/or communication with the ECM is therefore not established for some prominent DGCs, such as those seen in microvascular endothelial cells and the CP. However, negative results are not necessarily conclusive, and the failure to detect dystroglycan in some tissues might be due to technical reasons. Furthermore, some biochemical methods are of limited use in heterogeneous tissues with a cell-specific expression of DGC proteins. It is important to note that in neurons and astrocytes, as well as kidney tubular epithelial cells, the localization of the DGC precisely matches the presence of a basal lamina, suggesting that communication with the ECM via α-DG is essential for specifying the subcellular localization of the DGC. This feature might, for example, explain the

remarkably specific targeting of the DGC in astrocytic endfeet. In the CP, the DGC is present basolaterally in epithelial cells, arguing against the presence of dystroglycan in this tissue.

The analysis of mutant mice has revealed that in all cell types coexpressing a dystrophin isoform with utrophin, compensatory up-regulation takes place in the absence of the homologue protein (Fig. 4), obscuring the interpretation of the analysis of single mutant mice. In contrast, these compensatory mechanisms do not take place in tissues expressing only dystrophin (glial endfeet) or only utrophin (CP epithelial cells, vascular endothelial cells). The analysis of double-mutant mice lacking dystrophin and utrophin is hampered by the severe phenotype, limited breeding capacity, and reduced life expectancy of these animals.

A major convergent finding emerging from the analysis of the DGC in non-muscle tissue is that syntrophins require the presence of either dystrophin or utrophin for assembly of the DGC and membrane localization. Owing to the presence of several 'simple' DGCs containing a reduced number of proteins, a mandatory association of β 2-syn with utrophin, as well as α -DB, α 1-syn, and β 1-syn with dystrophin, has been demonstrated in three distinct tissues (Fig. 4). The role of β -DB is less clear since it does not disappear from the CP of utrophin^{0/0} mice and associates with Dp71 in the absence of utrophin in the kidney. Therefore, although dystroglycan is a key member of the DGC, interacting with signaling proteins, dystrophin and utrophin appear to be essential for the formation of the complex, with very few exceptions so far [234].

Several membrane-associated proteins have been identified, notably AQP4 and Kir4.1, which depend on the DGC for proper targeting and localization. However, no generalization is possible since major transporters, such as members of the ABC transporter or the glucose transporter families, which colocalize with the DGC in several tissues, are not affected in mutant mice. It is therefore difficult to predict functional deficits that might arise from an altered expression of DGC proteins. However, the existence of functional redundancy between dystrophin isoforms, dystrobrevin, and utrophin might represent a strong stimulus for exploiting further compensatory mechanisms to alleviate the symptoms of muscle dystrophy.

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