Published in final edited form as:

Brain Res. 2008 July 7; 1218: 21–34. doi:10.1016/j.brainres.2008.04.071.

Dystrophin and utrophin isoforms are expressed in glia, but not neurons, of the avian parasympathetic ciliary ganglion[★]

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Abstract

Muscular dystrophy patients often show cognitive impairment, in addition to muscle degeneration caused by dystrophin gene defects. The cognitive impairments lead to speculation that the dystrophin protein family may play a key role at neuronal synapses. Dystrophin regulates the stability of selected GABA_A receptor subtypes and α 3-containing nicotinic acetylcholine receptors (nAChRs) at a subset of central GABAergic and peripheral sympathetic nicotinic neuron synapses. Similarly, utrophin, the autosomal homologue of dystrophin, is not required for clustering but indirectly stabilizes muscle-type nAChRs at the neuromuscular junction. We examined dystrophin and utrophin expression and localization in the avian parasympathetic ciliary ganglion (CG) to determine whether these proteins play a general role at neuronal nicotinic synapses. We have determined that full-length utrophin and dystrophin and the short dystrophin isoform Dp116 are the major isoforms expressed in the CG based on immunoblotting and immunolabeling. Unexpectedly, the cytoskeletal proteins were not detected at nicotinic synapses or in CG neurons. They are expressed in myelinating and non-myelinating Schwann cells. Further, utrophin expression developmentally precedes that of dystrophin. The proteins show partially overlapping distributions, but also differential accumulation along the surface membrane of Schwann cells adjacent to neuronal somata versus axonal processes. Our findings are consistent with reports that dystrophin protein family members function in the maintenance of cell–cell interactions and myelination by anchoring the Schwann cell surface membrane to the basal lamina. In contrast, our results differ from those in skeletal muscle and a subset of sympathetic neurons where utrophin and dystrophin localize at nicotinic synapses.

Keywords

Dystrophin; Utrophin; Schwann cell; Parasympathetic ciliary ganglion; neuron; Nicotinic acetylcholine receptor; Synapse

1. Introduction

Dystrophin is an essential cytoskeletal protein that is mutated or absent in Muscular Dystrophy, a progressively disabling muscle degenerative disorder (Hoffman et al., 1987; Emery, 1988). Muscular Dystrophy patients also have nervous system involvement resulting in non-progressive cognitive impairments (Lidov, 1996; Blake and Kroger, 2000; Mehler, 2000). The mdx mutant mouse, a dystrophic disease model that lacks dystrophin, also shows cognitive impairments (Vaillend et al., 2004; Anderson et al., 2004). This disease highlights the

[★]This research was funded by NIH grant NS 21725 to MHJ.

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importance of defining the role of the dystrophin protein family in the vertebrate nervous system.

The dystrophin protein family includes multiple dystrophin and utrophin isoforms that have differential expression patterns and functions in skeletal muscle and the central and peripheral nervous systems (Table 1). Dystrophin and the closely related cytoskeletal protein utrophin are encoded by separate genes, but share great similarity in their protein interaction domains (Fig. 1A; reviewed in Blake et al., 1996). Several dystrophin and utrophin full-length and shorter isoforms are generated by different promoters and alternative splicing (Fig. 1A) (Feener et al., 1989; Gorecki et al., 1992; Byers et al., 1993; Lidov et al., 1995; Wilson et al., 1999; Jimenez-Mallebrera et al., 2003). Diverse functions of the variants stem from their ability to organize related but distinct membrane-bound glycoprotein complexes consisting of isoforms of syntrophin, dystroglycan, sarcoglycan, and dystrobrevin (Kramarcy et al., 1994; Yang et al., 1995; Rivier et al., 1999; Banks et al., 2003). The glycoprotein complex components interact with and localize specific ion channels and signaling molecules to precise membrane domains (Gee et al., 1998; Connors and Kofuji, 2002; Saito et al., 2003; Amiry-Moghaddam et al., 2004; Connors et al., 2004; Haenggi et al., 2004; Johnson et al., 2005).

The separate functions and localizations of dystrophin and utrophin are well-defined in skeletal muscle. Dystrophin localizes along the entire myofiber sarcolemma, outside of the neuromuscular junction (nmj) (reviewed in Banks et al., 2003). Its interactions with the membrane-bound glycoprotein complex link the intracellular actin cytoskeleton to the extracellular matrix and support the mechanical integrity of the myofiber (Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Carpenter et al., 1990; Kramarcy et al., 1994). The dystrophin glycoprotein complex also organizes signaling cascades, as demonstrated by abnormal intracellular calcium levels and nitric oxide signaling in dystrophic muscle (Shiao et al., 2004; Johnson et al., 2005; Wang et al., 2005). At the nmj, dystrophin colocalizes with sodium channels at the troughs, but is excluded from the nicotinic acetylcholine receptor (nAChR)-rich crests of the junctional folds (Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Byers et al., 1991; Bewick et al., 1992; Gee et al., 1998). In contrast, utrophin is concentrated at the nmj and colocalizes with nAChRs (Khurana et al., 1991; Ohlendieck et al., 1991; Bewick et al., 1992). Utrophin is not required for nAChR clustering, but its targeted deletion in mice causes a decline in nAChR levels at the nmj, suggesting that utrophin functions in their stabilization (Deconinck et al., 1997; Grady et al., 1997; Slater et al., 1997; Banks et al., 2003).

Similarly, full-length dystrophin promotes the stabilization of postsynaptic receptors in neurons. Dystrophin colocalizes with selective GABA_A receptor subunits at a subset of inhibitory GABAergic synapses in the CNS (Knuesel et al., 1999, 2001; Fritschy et al., 2003). Dystrophin also localizes to a subset of α 3-nAChR-rich postsynaptic sites in sympathetic superior cervical ganglion (SCG) neurons (De Stefano et al., 1997; Zaccaria et al., 2000; Del Signore et al., 2002). Although dystrophin is not required for cluster formation, there are decreases in the number of GABA_A receptor and α 3-containing nAChR clusters and impaired inhibitory synaptic transmission in *mdx* and dystrobrevin mutant mice (Levi et al., 2002; Knuesel et al., 1999; Zaccaria et al., 2000; Del Signore et al., 2002; Anderson et al., 2003; Grady et al., 2006; Kueh et al., 2008). These studies define a specific neural function for dystrophin and its associated glycoprotein complex.

Dystrophin and utrophin isoforms also function in non-neuronal cells of the vertebrate nervous system (Table 1), and disruption of these non-neuronal functions may contribute to the neurological symptoms associated with Muscular Dystrophy (Moizard et al., 1998; Nico et al., 2004). Specifically, the short dystrophin isoform, Dp71, is expressed at glial endfeet that surround blood vessels in the CNS and is essential for maintenance of blood-brain barrier

integrity (Claudepierre et al., 2000; Connors and Kofuji, 2002; Dalloz et al., 2003; Amiry-Moghaddam et al., 2004; Connors et al., 2004). Similarly, full-length utrophin is expressed in glia, blood vessels, choroid plexus and pia mater (Khurana et al., 1992; Kamakura et al., 1994; Imamura and Ozawa, 1998; Claudepierre et al., 2000; Knuesel et al., 2000). The dystrophin isoform Dp116 and its associated glycoprotein complex are required for maintenance of peripheral myelination, as seen in animal models of dystrophy and a dystrophic patient with loss of Dp116 (Matsumura et al., 1993; Comi et al., 1995; Saito et al., 1999; Imamura et al., 2000; Masaki et al., 2001; Sherman et al., 2001; Saito et al., 2003; Cai et al., 2007). Cognitive deficits and peripheral neuropathy in dystrophic patients are consistent with both synaptic and glial functions of dystrophin protein family members.

To gain further insights into the neural function of dystrophin and utrophin isoforms, we characterized their expression in a vertebrate parasympathetic ganglion. We speculated that the roles of utrophin and dystrophin as stabilizers of nAChR clusters in skeletal muscle and sympathetic neurons may be shared at other nicotinic synapse types. We show here that three major isoforms, full-length utrophin and dystrophin and Dp116, are expressed in the avian ciliary ganglion (CG). The cytoskeletal proteins were not detected at nicotinic synapses or in CG neurons, however. They were detected in myelinating and non-myelinating Schwann cells. Further, we show differences in temporal onset of expression and subcellular localization of utrophin and dystrophin proteins. Our findings are consistent with a global role for these cytoskeletal proteins in Schwann cells, but not at neuronal nicotinic synapses.

2. Results

2.1. Antibody cross-reactivity with chicken dystrophin and utrophin

For our characterization of dystrophin and utrophin expression in neurons and glia of the avian embryonic CG, we first tested multiple antibodies raised against mammalian dystrophin and utrophin for crossreactivity with the chicken proteins. The epitopes for all five antibodies used are shown in Fig. 1A. MANDRA1 should recognize any dystrophin isoform, while MANDYS8 will only bind full-length dystrophin. Mupa-3 should recognize any utrophin isoform, Mupa-2 should recognize all except Up71, and NCL-DRP2 will only bind full-length utrophin and N-utrophin.

We started with chicken skeletal muscle as a positive control in immunolabeling and immunoblotting analyses. The localization of dystrophin and utrophin protein within skeletal muscle has been well characterized by immunolabeling studies in other species. Typically, dystrophin accumulates at the myofiber surface membrane but not the nmj, whereas utrophin is concentrated at the nmj and blood vessels (Khurana et al., 1991; Ohlendieck et al., 1991; Bewick et al., 1992; Rivier et al., 1997). We found two dystrophin (MANDYS8 and MANDRA1) and three utrophin (NCL-DRP2, Mupa-3 and Mupa-2) antibodies that labeled in an appropriate spatial distribution in chicken skeletal muscle, suggesting that these antibodies show specific cross-reactivity. MANDYS8 and MANDRA1 immunostaining were seen around the periphery of the entire myofiber but were largely excluded from the nmj (Figs. 1B,C). In contrast, NCL-DRP2, Mupa-3 and Mupa-2 immunostaining was concentrated at the nmj, strongly colocalizing with nAChR labeling (Figs. 1D,E and Mupa-2 data not shown). Utrophin immunostaining was also present at blood vessels (Figs. 1D,E). As a test for specificity of the immunostaining pattern, omission of the primary antibodies showed only background levels of labeling in muscle (Figs. 1C,E). As a direct test of specific crossreactivity of the primary antibodies, immunoblotting of chicken skeletal muscle protein showed that the antibodies recognized a single major band of the expected size for full-length dystrophin and utrophin (Fig. 2A). Based on their specific crossreactivity in chicken muscle, we then used the antibodies to define the expression and spatial distribution of dystrophin and utrophin isoforms in the embryonic chicken CG.

2.2. Multiple dystrophin and utrophin isoforms are expressed in the CG

Immunoblot analyses suggest that three dystrophin and utrophin isoforms are predominantly expressed in the chicken CG. Full-length dystrophin and Dp116 were detected as major bands using MANDRA1 and MANDYS8 antibodies (Fig. 2A). Full-length utrophin was detected as a major band using NCL-DRP2, Mupa-2 and Mupa3 antibodies. No bands were apparent in control lanes with the primary antibodies omitted (data not shown).

Developmental comparison of expression levels at embryonic day (E) 12 and E19 showed a 20% reduction in utrophin levels at the older age (normalized band intensity 2.31 vs. 2.92, $p < 0.005$, Student's t-test, $n = 3$; Fig. 2B). In contrast, levels of the dystrophin isoform Dp116 showed a 3-fold increase at E19 compared with E12 (normalized band intensity 2.88 vs 0.88, $p < 0.005$, $n = 5$; Fig. 2B). Full-length dystrophin also showed a small but significant increase (normalized band intensity 1.29 vs. 0.90, $p < 0.05$, $n = 5$; Fig. 2B). Data were normalized to β -tubulin levels as a loading control.

2.3. Dystrophin and utrophin proteins are expressed in Schwann cells, not neurons, in the CG

Next we used immunolabeling to define the localizations of dystrophin and utrophin proteins in the CG (Fig. 3). We used both amino- and carboxy-terminal antibodies to ensure detection of full-length dystrophin and utrophin, as well as the Dp116 splice variant. To localize the dystrophin and utrophin proteins relative to neurons, and in particular, nicotinic cholinergic synapses, we used double labeling immunofluorescence with antibodies to several different synaptic proteins: synaptic vesicles, as a marker of presynaptic terminals, $\alpha 3$ -nAChRs that are concentrated in the postsynaptic membrane, and $\alpha 7$ -nAChRs that localize perisynaptically on somatic spines in CG neurons (Jacob and Berg, 1983; Jacob et al., 1986; Williams et al., 1998; Coggan et al., 2005). We stained only the surface pool of $\alpha 3$ -nAChRs to mark nicotinic postsynaptic sites and both the surface and internal biosynthetic pools of $\alpha 7$ -nAChRs to mark neurons. No significant dystrophin or utrophin labeling was seen to be associated with the neurons, nAChR surface clusters, or synapses in the CG (Fig. 3). We found a predominance of distinct red and green fluorescence labeling and the fluorescent intensity profiles did not co-vary, indicating the lack of co-localization. These findings suggest that no dystrophin or utrophin isoforms are expressed in CG neurons, at least none that are recognized by the antibodies used for this study.

In contrast to the lack of neuronal labeling, dystrophin and utrophin immunostaining was present in small cells that surround neuronal somata and axonal processes in the CG (Fig. 3). Their location and size suggests their identity as Schwann cells (Landmesser and Pilar, 1972; Jacob, 1991). Omission of the dystrophin and utrophin primary antibodies resulted in only background labeling, suggesting specificity of the staining (Figs. 3M,N). Further, different Schwann cell types are associated with the two distinct neuron populations of the CG, ciliary and choroid neurons. Schwann cells ensheathing the ciliary neuron somata and axons are myelinating, whereas those that surround the choroid neuron somata and axons are not (Fig. 4, Hess, 1965). To confirm the identity of the dystrophin and utrophin immunopositive cells in the CG and determine whether both myelinating and non-myelinating Schwann cells express these proteins, we used double-labeling immunofluorescence with a myelin basic protein (MBP) antibody (Figs. 4B–E). Dystrophin and utrophin labeling overlapped with MBP staining in Schwann cells surrounding the ciliary neuron somata and axonal processes (Figs. 4C,E). As expected, MBP staining was absent from the quadrant of the ganglion containing choroid neurons (Figs. 4B,D). Dystrophin and utrophin labeling was also present in the MBP-immunonegative Schwann cells that surround the choroid neurons.

Because of the close physical contact between neurons and Schwann cells, we used immunolabeling of CG dissociated cell cultures to determine whether dystrophin and utrophin are expressed near the surface membrane of both or only one of the two cell types. Neither dystrophin nor utrophin labeling were detected in neuronal cell bodies marked by Tau- and Tuj1 staining. Instead, the labeling was concentrated along non-neuronal cell surface membrane regions and was also present intracellularly (Fig. 5). Taken together, the immunolabeling and immunoblot data suggest that dystrophin and utrophin proteins are expressed in myelinating and non-myelinating Schwann cells, but not in neurons, of the parasympathetic CG.

2.4. Differences in onset of expression and localization of Dp116, dystrophin and utrophin in Schwann cells

We found differences in the time course of expression and localization of dystrophin and utrophin proteins in CG Schwann cells during differentiation and myelination. Landmark stages of CG development are: E4.5–8, the CG neurons are being innervated and extending axons, E8.5–14, synaptic connections with target tissues are being established, and E14, myelination of the ciliary neuron soma and axon begins (Landmesser and Pilar, 1972, 1976; Meriney and Pilar, 1987; Pilar et al., 1987; Jacob, 1991). We found that utrophin expression precedes that of the dystrophin isoforms; it is seen by immunolabeling at E6, whereas dystrophin proteins are not detectable until E8.5 (Fig. 6). The cytoskeletal proteins are expressed in the Schwann cells throughout the remainder of embryonic development, and their levels change with maturation (Fig. 6). Utrophin immunostaining increases up to E13.5, but the levels appear lower at E20, resembling the decrease seen by immunoblotting (Fig. 2B and Fig. 6). In comparison, dystrophin isoform levels increase developmentally through E20, in agreement with our immunoblotting results (Fig. 2B and Fig. 6).

Utrophin and dystrophin proteins accumulate along the surface membrane of the Schwann cells and show a generally similar immunostaining pattern up to E13.5. At later developmental stages of maturation and myelination, however, we found differences in the localization of utrophin and dystrophin relative to one another. Double-labeling immunofluorescence indicated partial co-localization, as well as distinct regions of utrophin and dystrophin staining along the Schwann cell surface at E17 and E20 (Fig. 7A). Accordingly, fluorescence intensity profiles showed regions of strong covariance adjacent to regions of distinct utrophin or dystrophin labeling (Fig. 7A1). For quantitative assessments, we measured fluorescent pixel intensities of the surface labeling in Schwann cells adjacent to neuronal somata versus those adjacent to axonal processes. We found highest utrophin labeling in Schwann cells encapsulating neuronal somata as compared to those ensheathing axons, as illustrated by comparison of pixel intensity distributions (Fig. 7B). In contrast, dystrophin showed the reciprocal pattern, highest labeling in axon-associated versus neuronal soma-associated Schwann cells (Fig. 7C). This localization is consistent with reports that the Dp116 glycoprotein complex is required for maintenance of peripheral myelination (Comi et al., 1995; Saito et al., 2003; Cai et al., 2007).

3. Discussion

We show here that utrophin and dystrophin, cytoskeletal proteins implicated in the stabilization of nAChR clusters in skeletal muscle and sympathetic neurons, are also expressed in another nicotinic preparation, the avian parasympathetic ciliary ganglion. Utrophin and dystrophin proteins were not detectable at nicotinic synapses or in neurons of the CG, however. The proteins are expressed in myelinating and non-myelinating Schwann cells. We have identified full-length dystrophin and utrophin, and Dp116 as the major isoforms expressed in the CG. We found differences in their developmental time course of expression and localization,

suggesting distinct functions. Utrophin expression precedes that of the dystrophin isoforms by several days during early neuron and Schwann cell interactions in the embryonic CG. At later stages of Schwann cell maturation and myelination, utrophin levels decrease, whereas Dp116 and full-length dystrophin levels continue to increase. Further, utrophin shows greater accumulation in Schwann cells surrounding neuronal somata, whereas dystrophin isoforms accumulate more in axon-ensheathing Schwann cells. Our results are consistent with a global role for utrophin and dystrophin proteins in maintenance of intercellular interactions and myelination in Schwann cells of the vertebrate PNS.

We did not detect utrophin or dystrophin proteins at CG nicotinic synapses using multiple antibodies that recognize distinct epitopes. These results contrast with the presence of full-length utrophin at the nmj, where it co-localizes with muscle-type nAChRs and functions in their stabilization (Khurana et al., 1991; Ohlendieck et al., 1991; Deconinck et al., 1997; Grady et al., 1997; Slater et al., 1997; Banks et al., 2003). Surprisingly, our results at $\alpha 3$ -containing nAChR synapses in CG neurons also differ from reports that full-length dystrophin is present at a subset of $\alpha 3$ -nAChR-containing synapses in SCG neurons (De Stefano et al., 1997; Zaccaria et al., 2000; Del Signore et al., 2002). There are several possible explanations for this difference. Parasympathetic CG and sympathetic SCG neurons may differ in at least some nicotinic synaptic components. Alternatively, dystrophin may be expressed in CG neurons but was not detected, possibly because the antibodies we used have low cross-reactivity with the chicken protein or the epitopes are inaccessible. This possibility does not seem likely, however, because we readily detected dystrophin labeling in chicken skeletal muscle and Schwann cells. Moreover, we used two different antibodies that recognize distinct epitopes in the dystrophin protein (Fig. 1A). The rodent SCG studies used different antibodies (not commercially available). Precedence exists for different antibodies to utrophin giving contradictory results in neurons (Kamakura et al., 1994; Knuesel et al., 2001). Interestingly, dystrophin was only detected at a subset (15%) of the nicotinic synapses in the SCG (Zaccaria et al., 2000). Moreover, dystrophin is also present at a subset of adherens junctions and in Schwann cells of the SCG (De Stefano et al., 1997). Taken together, a general role for dystrophin and utrophin at neuronal nicotinic synapses seems unlikely based on their absence from CG synapses and presence at only a subset of SCG synapses.

Although not detected in CG neurons, full-length dystrophin and utrophin and Dp116 are expressed in myelinating and non-myelinating Schwann cells within the ganglion. We found differences in the temporal onset of expression; utrophin expression preceded that of dystrophin isoforms during stages of early neuron and Schwann cell interactions in the embryonic CG. At later stages of Schwann cell maturation and myelination, utrophin levels decrease, whereas Dp116 and full-length dystrophin levels continue to increase. These data resemble the developmental expression patterns of dystrophin and utrophin in human brain and skeletal muscle (Lin and Burgunder, 2000; Radojevic et al., 2000; Sogos et al., 2002).

We also found partially overlapping as well as differential localizations for these proteins in Schwann cells of the CG; utrophin shows greater accumulation in Schwann cells surrounding neuronal somata, whereas dystrophin isoforms accumulate more in axon-ensheathing Schwann cells. Accumulating evidence suggests that different dystrophin and utrophin isoforms have distinct functions in glial cells. The different isoforms organize related but distinct membranebound glycoprotein complexes. These multimolecular complexes have two major functions: 1) provide structural anchorage and adhesion by linking the actin cytoskeleton to the basal lamina; and 2) localize specific ion channels and signaling molecules to precise surface membrane regions (Matsumura et al., 1993; Yamada et al., 1994; Saito et al., 1999; Imamura et al., 2000; Masaki et al., 2001; Haenggi et al., 2004). In peripheral nerve, Dp116 and full-length utrophin provide structural support to the outer surface membrane of Schwann cells by linking the actin cytoskeleton and basal lamina (Yamada et al., 1994; Saito et al.,

1999; Imamura et al., 2000; Saito et al., 2003). Demyelinating neuropathy has been seen in dystrophic patients with loss of Dp116 (Comi et al., 1995), and myelin instability occurs in animal models in which the dystrophin complex is disrupted (Saito et al., 2003; Cai et al., 2007). Interestingly, the Dp116 complex interacts with the cholesterol transporter that is mutated in Tangier disease, which has a demyelinating peripheral neuropathy phenotype (Züchner et al., 2003; Albrecht et al., 2008). Our findings are consistent with a role for dystrophin and utrophin proteins in Schwann cell–neuron interactions and myelination. Based on their differential expression patterns in CG Schwann cells, it would be interesting to test for specific roles of these two proteins in localizing ion channels and signaling molecules at the surface of Schwann cells adjacent to the synapse-rich soma versus axonal processes of CG neurons.

Cognitive deficits and demyelinating neuropathy have both been seen in dystrophic patients (Comi et al., 1995; Lidov, 1996; Blake and Kroger, 2000; Mehler, 2000). The cognitive impairments lead to speculation that the dystrophin protein family may play a key role at neuronal synapses. Best evidence for such a role comes from reports that full-length dystrophin regulates the stability of selective GABA_A receptors and α 3-containing nAChRs at a subset of central GABAergic and peripheral sympathetic nicotinic synapses (Knuesel et al., 1999, 2001; Zaccaria et al., 2000; Del Signore et al., 2002; Levi et al., 2002; Fritschy et al., 2003). However, our findings do not support a postsynaptic role for dystrophin and utrophin proteins at nicotinic synapses in parasympathetic CG neurons. On the other hand, our data are consistent with accumulating evidence for a key role for dystrophin protein family members in glial cells in the vertebrate nervous system.

4. Experimental procedures

4.1. Chicken embryos

White Leghorn embryonated chicken eggs (Charles River/Spafas, North Franklin, CT) were maintained until use in a humidified forced-draft 37 °C incubator with automated rocking motion. Embryo staging followed the Hamburger and Hamilton (1992) classification scheme. All experiments conformed to guidelines established by the Institutional Animal Care and Use Committee (IACUC).

4.2. Immunoblotting

Embryonic chicken skeletal muscle and CGs (E12 and E19) were dissected into 1× SDS-PAGE sample buffer containing 5 mM EDTA and a general protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) on ice. The samples were solubilized by Dounce homogenization, boiled for 10 min at 100 °C, spun down, and separated on 6% SDS-polyacrylamide gels. Equal numbers of ganglia (10 CG equivalents) were added per lane. For developmental immunoblot analyses, CGs (E12 and E19) were solubilized in T-PER protein extraction reagent containing protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL) and spun down. 4× SDS-PAGE sample buffer (Boston BioProducts, Worcester, MA) was added, and samples were boiled for 10 min at 100 °C and separated on 6% SDS-polyacrylamide gels. Equal amounts of protein (20 µg) were loaded per lane. Separated proteins in the gels were transferred electrophoretically onto nitrocellulose membranes. Blots were treated with 5% dry milk in TNT buffer (10 mM Tris, pH 7.4; 150 mM NaCl; 0.5% Triton-X 100) for 1.5 h to block non-specific binding, then probed with MANDRA1 (1:3,000) and MANDYS8 (1:2,000) monoclonal antibodies to dystrophin (Sigma), NCL-DRP2 monoclonal antibody to utrophin (1:20, NovoCastra Laboratories, Newcastle-upon-Tyne, U.K.), Mupa2 (1:500) or Mupa3 (1:15,000) polyclonal antibodies to utrophin (kind gift of Dr. Cecilia Jimenez-Mallebrera, Imperial College, London), or monoclonal antibody to β -tubulin (1:5,000, Covance, Princeton, NJ) in 5% dry milk in TNT buffer for 2 h, washed 3 times for 10 min with TNT, incubated

with horse-radish peroxidase-conjugated anti-mouse or anti-rabbit antibodies at 1:3000 (Amersham Biosciences, Amersham, U.K.) for 1 h, washed 3 times for 10 min with TNT, and visualized using enhanced chemiluminescence (PerkinElmer, Waltham, MA). All immunoblot procedures were carried out at room temperature. Immunoblot band densities were quantified using Kodak Molecular Imaging Software.

4.3. Immunohistochemistry

Embryonic chicken skeletal muscles at E20 and CGs at selected ages ranging from E5 to E20 were dissected into phosphate buffered saline (PBS) pH 7.4 on ice. To only label surface α 3-nAChRs (and avoid labeling the large internal biosynthetic pool), the freshly dissected intact CGs were treated with 1 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN) for 10 min, rinsed and incubated with mAb35 (Developmental Studies Hybridoma Bank, Iowa City, IA) at 1:100 in PBS for 1 h at 4 °C on a shaker. CGs were then washed 2 times for 15 min with PBS, fixed for 30 min in 1% paraformaldehyde, incubated 15 min each in 10%, 20%, and 30% sucrose as a cryoprotectant, embedded in Tissue-Tek OCT (Electron Microscopy Sciences; Hatfield, PA), and frozen with liquid nitrogen. Tissues that were not pre-labeled in this manner were fixed for 30 min in 1% paraformaldehyde and processed as above for embedding and freezing. Frozen sections 9 μ m thick were cut on a Leica Microsystems (Bannockburn, IL) CM1900 cryostat. Frozen sections were rinsed with PBS, blocked in 10% normal horse serum in PBS pH 7.4 for 30 min and incubated with primary antibodies for 45 min. Primary antibodies used were: mouse monoclonal antibodies against dystrophin (MANDRA1 at 1:300 and MANDYS8 at 1:100; Sigma), utrophin (NCL-DRP2 at 1:5–1:10; Novocastra), and synaptic vesicles (SV2 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA or synapsin 1:500; American Qualex, San Clemente, CA), and polyclonal antisera to utrophin (Mupa-2 at 1:50–1:100 and Mupa-3 at 1:1000) and myelin basic protein (MBP) (New England Biolabs, Ipswich, MA). Alternatively, primary antibodies were omitted as a negative control. Secondary reagents used were: Alexa-Fluor-488, –555 conjugated secondary antibodies (Molecular Probes-Invitrogen, Eugene, OR) and Cy3-streptavidin D (Jackson Labs, West Grove, PA). We labeled α 7-nAChRs with biotinylated α -bungarotoxin (α -btx) at 1:100 (Molecular Probes-Invitrogen). After incubation with the primary antibodies or α -btx, sections were washed with PBS pH7.4 for 15 min, incubated with secondary antibodies or streptavidin for 45 min, washed with PBS pH7.4 for 15 min, dehydrated with methanol, covered with Vectashield (Vector Labs, Burlingame, CA) and mounted with glass coverslips (Corning, Lowell, MA). All steps were carried out at room temperature. All images were captured using a Zeiss Axioskop microscope (Zeiss, Thornwood, NY) with a 40 $\times$ air or 63 $\times$ oil objective, a Spot camera and software (Diagnostic Instruments, Sterling Heights, MI). Image analysis was done in Adobe Photoshop, version 6.0.

To quantify immunolabeling levels in Schwann cells surrounding the neuronal soma versus axons, we assessed pixel intensity profiles along ~10 μ m length segments of the brightest labeled surface regions per Schwann cell using Image J software (<http://rsb.info.nih.gov/ij/>). For each immunolabel, we analyzed pixel intensities for one line segment per Schwann cell and 10–20 cells from 5 separate embryos. The pixel intensities of sampled surface segments were binned into incremental groups of 5 pixel intensity steps (e.g. 0–5, 5–10 etc., up to saturation) for images acquired with Spot camera (dynamic range 0–255). We calculated the percentage of pixels that belonged to each pixel intensity category and plotted the relative frequency distributions.

4.4. Ciliary ganglion dissociation

E13 CGs were dissociated by incubation with collagenase A (1 mg/mL; Roche) in calcium-free avian basal salt solution at 37 °C for 30 min, followed by brief trituration (Temburni et al., 2004). Cells were plated on laminin-coated glass coverslips in 24-well culture plates and

maintained at 37 °C for 48 h in minimum essential medium (MEM; Gibco-Invitrogen, Eugene, OR) supplemented with 10% horse serum and 3% chick eye extract. Cells were fixed for 30 min in 2% paraformaldehyde, permeabilized for 2 min in 0.05% saponin, and pre-blocked with 0.25% cold water fish gelatin (Sigma) in PBS for 30 min. Primary antibodies against dystrophin (Mandra1, 1:300), utrophin (Mupa3, 1:1000), β -tubulin (1:500, Covance), and tau (1:500; Dako, Glostrup, Denmark) were applied for 1 h. Cells were then washed with 0.125% gelatin in PBS for 15 min, incubated with fluorophore-conjugated secondary antibody for 1 h, washed with PBS for 15 min, dehydrated with methanol, and mounted with Vectashield on glass slides (Fisher Scientific, Waltham, MA).

Acknowledgments

We would like to thank Dr. Cecilia Jimenez-Mallebrera (Imperial College, London) for the generous gift of the Mupa-2 and Mupa-3 antibodies, and Dr. Dominique Mornet (Université de Montpellier, France) for dystrophin and utrophin antibodies.

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Fig. 1.

Characterization of dystrophin and utrophin antibody cross-reactivity with the chicken proteins by immunolabeling skeletal muscle as a positive control. A: Schematic representation of dystrophin and utrophin isoforms and their domains. These ~400 kDa proteins both consist of an amino-terminal actin binding domain, a large number of spectrin repeats, followed by a cysteine-rich domain and a carboxy-terminus with multiple protein binding domains (Koenig et al., 1988; Love et al., 1989; Tinsley et al., 1992; Ponting et al., 1996). ABD, actin binding domain; oval, spectrin repeat; CR, cysteine-rich domain; C, carboxy-terminus. The binding sites of monoclonal and polyclonal antibodies to dystrophin and utrophin are indicated. B–E: Double labeling immunofluorescence of E20 chicken skeletal muscle frozen sections.

Acetylcholine receptors (red, mAb35), stained as a marker of the nmj in all panels. Insets: representative nmjs at 2-fold higher magnification. B: dystrophin (green, MANDYS8) and C: dystrophin (green, MANDRA1) immunostaining are seen extending along the surface of each myofiber but largely excluded from the nmj. D: utrophin (green, NCL-DRP2) and E: utrophin (green, Mupa-3) show strong overlap with nAChRs (red) at the nmj, as indicated by the predominance of yellow immunofluorescent staining. D, E: Arrows=blood vessels immunostained for utrophin. Omission of primary dystrophin (C) and utrophin (E) antibody shows only background staining of skeletal muscle. Scale bar=10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Immunoblot analyses of dystrophin and utrophin isoforms expressed in the chicken CG during development. A: E12 CG blots probed with the dystrophin antibodies MANDRA1 and MANDYS8 and utrophin antibodies Mupa2, Mupa3, and NCL-DRP2, showing full-length dystrophin (427 kDa), Dp116 (116 kDa) and full-length utrophin (395 kDa) as major bands. E12 skeletal muscle (Mus), as a positive control, showed full-length dystrophin and utrophin bands. B: E12 and E19 CG blots indicate developmental changes in levels of utrophin and dystrophin isoforms. At E19, utrophin levels showed reductions, whereas full-length dystrophin and Dp116 levels were increased relative to E12 values. $*=p<0.05$; $**=p<0.005$, Student's t-test, $n=3-5$ separate experiments. Blots were probed with antibodies to dystrophin

(MANDRA1), utrophin (Mupa3) and β -tubulin as a loading control. Molecular weight markers = size in kDa of Precision Plus Prestained Marker (Biorad).

Fig. 3.

Immunolocalization of utrophin and dystrophin relative to CG neurons and synaptic proteins. Double-label immunofluorescence of E18–21 chicken CG frozen sections. Distribution of utrophin (green) and dystrophin (green) relative to: (A–D) presynaptic terminals marked by labeling for synaptic vesicles (red), (E–H) $\alpha 3$ -nAChR surface clusters (red) that are concentrated at postsynaptic sites, and (I–L) $\alpha 7$ -nAChR perisynaptic surface clusters and internal biosynthetic pool (red) that mark the CG neurons. Neither utrophin nor dystrophin isoforms co-localize with the three different neuron-specific proteins. Instead, utrophin and dystrophin labeling is restricted to cells that surround neuronal cell bodies, axon processes and presynaptic terminals. Left Insets in E–H: 2-fold higher magnification views of boxed regions. Right Insets: Fluorescent intensity profiles show that the labeling does not co-vary for utrophin and dystrophin proteins (green) with $\alpha 3$ -nAChRs (red). A, C, D: synapsin; B: SV2; A, E, I: utrophin (N-terminus, NCL-DRP2); B, F, J: utrophin (C-terminus, Mupa-3); C, G, K: dystrophin (spectrin-repeat region, MANDYS8); D, H, L: dystrophin (C-terminus, MANDRA1). Omission of primary antibody for utrophin (M) and dystrophin (N) shows only background levels of CG staining. Scale bar=15 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Localization of utrophin and dystrophin isoforms relative to myelinating and non-myelinating Schwann cells. A: Bright field image of a hatchling (1 week old) chicken CG epon-embedded section (1 μm thick) stained with toluidine blue. The section shows that ciliary neuron somata and axons are myelinated (arrows), whereas choroid neuron somata and axons are not (arrowheads). The two different neuron types localize to distinct quadrants in the ganglion (A, B, D) and are readily distinguished by their distinct morphologies and size. Ciliary neurons have a larger diameter (30–50 μm), ovoid shape and eccentric nucleus, whereas choroid neurons have a smaller diameter (15–30 μm), round shape and centrally placed nucleus (A) (Marwitt et al., 1971). B–E: Double-label immunofluorescence of E20 chicken CG frozen sections. Distribution of utrophin (green, B, C) and dystrophin (green, D, E) relative to myelin basic protein (MBP, red; overlap, yellow) immunopositive Schwann cells that surround ciliary neuron somata and axons (arrows) and MBP-immunonegative Schwann cells that surround choroid neuron somata and axons (arrowheads). Utrophin and dystrophin labeling is present in both myelinating and non-myelinating Schwann cells. B, C: utrophin (N-terminus, NCL-DRP2); D, E: dystrophin (C-terminus, MANDRA1). Scale bars=15 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Immunolocalization of dystrophin and utrophin in CG dissociated cell cultures. Double-label immunofluorescence of dissociated E13 CG cell cultures. Dystrophin (A, green, MANDRA1) and utrophin (B, red, Mupa-3) staining is absent from neuronal somata (N) and processes (arrows), based on lack of overlap with the neuron-specific markers tau (A, red) and Tuj1 (B, green). In contrast, dystrophin and utrophin staining is present intracellularly and along surface membrane regions (arrowheads) of non-neuronal cells that are not labeled by tau or Tuj1. Scale bar=10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Differences in utrophin and dystrophin developmental time course of expression in the embryonic chicken CG. Immunofluorescence labeling of chicken CG frozen sections at selected stages of differentiation and myelination. Utrophin labeling is present at E6, whereas labeling for dystrophin, full-length and Dp116, is not detectable until E8.5. From E13.5 up to older ages, utrophin labeling does not appear to increase, whereas dystrophin levels do, resembling the developmental immunoblot results (Fig. 2B). Staining for the cytoskeletal proteins is concentrated along the surface membrane of Schwann cells that encapsulate neuronal cell bodies and axonal processes. Utrophin (NCL-DRP2), dystrophin (MANDRA1). Scale bar=15 μ m.

Fig. 7. Overlapping and differential distribution of utrophin and dystrophin proteins in Schwann cells. A: Left, double-label immunofluorescence of E17 CG frozen section showing partial colocalization and differential distribution of utrophin (red, Mupa3) and dystrophin (green, MANDRA1) along the Schwann cell surface. Numbered boxes indicate locations of pixel intensity measurements in A1 and A2. A1: Pixel intensity profile showing regions of dystrophin and utrophin covariance (solid boxes) and distinct labeling (dotted box). A2: Pixel intensity profile showing highest utrophin labeling (red) in Schwann cells ensheathing the neuronal soma, whereas dystrophin labeling (green) is highest in axon-associated Schwann cells. B, C: Immunofluorescence labeling for utrophin (B) (red, Mupa2) and dystrophin (C) (green,

MANDRA1) in E17 and E20 CG frozen sections. Schwann cells surrounding neuronal somata (arrows) and axon processes (arrowheads). Right panels: Frequency distribution graphs show the highest pixel intensity for utrophin labeling in neuronal soma-associated Schwann cells (dark blue lines), whereas dystrophin highest pixel intensity is in axon-ensheathing Schwann cells (light blue lines) (see below). Dashed vertical lines indicate the median intensity values. Scale bar=15 μ m. For the quantitative assessments, the fluorescence pixel intensities were measured along 10 μ m length segments of labeled surface regions ($n=10-20$ Schwann cells, and 5 embryos for each immunolabeling experiment). The values were binned into incremental groups of 5 pixel intensity steps (from 0-5, 5-10, ..., up to saturation). The percentage of pixels that belonged to each pixel intensity category was calculated and the data plotted as a relative frequency distribution (B, C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Dystrophins and utrophins: nervous system expression and antibody reactivity

Isoform	CNS	PNS	Antibody reactivity
Dystrophin	Cortical and hippocampal pyramidal neurons (GABAergic synapses), purkinje cells, blood vessels ^{1,2,3}	Sympathetic SCG synapses ⁴	MANDYS8, MANDRA1
Dp260	Photoreceptor synapse in outer plexiform layer of retina ^{5,6}		MANDYS8, MANDRA1
Dp140	Glia and epithelial cells of developing brain ^{7,8}		MANDRA1
Dp116		Schwann cells and satellite cells ^{9,10,18}	MANDRA1
Dp71	Glia, blood vessels and Müller cells of inner limiting membrane of retina, hippocampal neurons ^{6,11,12,13}		MANDRA1
Utrophin	Cortical and brainstem neurons, blood vessels, astrocytes, choroid plexus, pia mater ^{2,13,14,15}	Schwann cells and satellite cells ^{9,10,18}	NCL-DRP2, Mupa-2, Mupa-3
G-utrophin	Neurons of cortex, basal ganglia, olfactory bulb ^{16,17}	Neurons of sensory ganglia ¹⁶	Mupa-2, Mupa-3

¹⁾References: Lidov et al., 1990;

²⁾Knuesel et al., 2000;

³⁾Levi et al., 2002;

⁴⁾De Stefano et al., 1997;

⁵⁾D'Souza et al., 1995,

⁶⁾Dalloz et al., 2001;

⁷⁾Lidov et al., 1995;

⁸⁾Lidov and Kunkel, 1997;

⁹⁾Byers et al., 1993;

¹⁰⁾Masaki et al., 2001;

¹¹⁾Jung et al., 1993;

¹²⁾Howard et al., 1998;

¹³⁾Claudepierre et al., 2000;

¹⁴⁾Khurana et al., 1992,

¹⁵⁾Kamakura et al., 1994;

¹⁶⁾Blake et al., 1995;

¹⁷⁾Jimenez-Mallebrera et al., 2003;

¹⁸⁾Occhi et al., 2005.