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ABERRANT DEVELOPMENT OF NEUROMUSCULAR JUNCTIONS IN GLYCOSYLATION-DEFECTIVE LARGE^{MYD} MICE

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Abstract

Mice deficient in the glycosyltransferase Large are characterized by severe muscle and central nervous system abnormalities. In this study, we show that the formation and maintenance of neuromuscular junctions in *Large^{myd}* mice are greatly compromised. Neuromuscular junctions are not confined to the muscle endplate zone but are widely spread and are frequently accompanied by exuberant nerve sprouting. Nerve terminals are highly fragmented and binding of α -bungarotoxin to postsynaptic acetylcholine receptors (AChRs) is greatly reduced. *In vitro*, *Large^{myd}* myotubes are responsive to agrin but produce aberrant AChR clusters, which are larger in area and less densely packed with AChRs. In addition, AChR expression on the cell surface is diminished suggesting that AChR assembly or transport is defective. These results together with the finding that *O*-linked glycosylation at neuromuscular junctions of *Large^{myd}* mice is compromised indicate that the action of Large is necessary for proper neuromuscular junction development.

Keywords

glycosylation; neuromuscular junction; skeletal muscle; dystroglycan

1. Introduction

Carbohydrates are uniquely assembled at synapses, such as the neuromuscular junction (NMJ), where specific glycans containing β -linked *N*-acetylgalactosamines (GalNAc) are concentrated and where many proteins at the NMJ are glycosylated including dystroglycan (DG), perlecan, NCAM, acetylcholine receptors (AChRs), agrin and MuSK [1-3]. These proteins play a role in the formation and maintenance of NMJs but the function of proper glycosylation of NMJ constituents remains largely unknown. AChR subunit proteins contain *N*-linked glycans in their N-terminal extracellular domains and glycosylation events have been implicated in receptor assembly, folding and transport [4-7]. Also, agrin, a molecule pivotally engaged in AChR cluster formation and maintenance is about one half sugar by weight [8]. It has heparan sulfate chains attached and contains potential *O*- and *N*-linked glycosylation sites [9]. Further, agrin possesses heparin-binding activity, which is, however, dispensable for NMJ formation [10]. Another key regulator of NMJ formation, the muscle-specific kinase MuSK contains two *N*-linked glycosylation sites and these sites appear to

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restrain ligand-independent tyrosine phosphorylation of MuSK and AChR clustering [11]. *N*-linked glycosylation of MuSK is dispensable for agrin-induced MuSK signaling. These data indicate that despite the glycosylation signature of synaptic proteins being highly diverse, the biological meaning of this phenomenon remains vastly unclear and *in vivo* models addressing this topic are needed to gain more insight into the role of glycosylation with respect to molecular, functional and structural integrity of the NMJ.

One of the glycoproteins mentioned above is dystroglycan (DG), which consists of α - and β -DG and which is an integral part of the dystrophin-associated glycoprotein complex (DGC). An important role of the DGC is to maintain the integrity of the muscle fiber sarcolemma and mutations in genes encoding members of the DGC are therefore associated with muscular dystrophy [12]. The DGC is expressed throughout the muscle membrane but specifically enriched at synaptic regions. At the postsynaptic muscle membrane the DGC is molecularly specialized. DGC proteins like the dystrophin-homologue utrophin, syntrophin β 2 and laminin α 4 are highly concentrated and mice with targeted deletions of these genes often display defects in NMJ stability and maintenance [13-15]. Studies on DG function and its role in NMJ formation have been hampered since targeted mutation of DG leads to lethality at E 6.5 [16]. Skeletal muscle devoid of DG isolated from chimeric mice, which were generated by injection of DG^{-/-} ES cells into wild-type blastocysts, have only few normal synapses, as most synapses are fragmented [17]. Myotubes derived from *in vitro* differentiated DG^{-/-} ES cells form agrin-induced AChR clusters but these clusters are less stable [18]. These data suggest that DG functions in the organization and stabilization of AChR clusters rather than in the formation of AChR clusters.

The glycosylation of DG has attracted attention due to the finding that various defects in putative or proven glycosyltransferases are associated with muscle disease. For instance, mice that carry a mutation in the *Large* gene (termed *Large*^{myd} mice) suffer from congenital progressive muscular dystrophy [19]. In addition, they show abnormal migration of central nervous system (CNS) neurons and have a defective visual system [20, 21]. The *Large* gene encodes a type 2 transmembrane protein with homology to an N-acetylglucosaminyltransferase [22], which is expressed throughout development with highest expression in brain, heart and muscle. Subsequently to the discovery that the molecular defect of the *Large*^{myd} mouse causes a glycosylation-dependent pathology, human diseases which are also characterized by muscular dystrophy and are accompanied by brain abnormalities and/or eye pathologies, such the human Fukuyama-type muscular dystrophy (FCMD) and muscle-eye-brain disease (MEB) have been found to be due to mutated glycosyltransferases [23]. As a common finding in both, murine *Large*^{myd} and human glycosylation-defective muscular dystrophies, α -DG has been found to be greatly hypoglycosylated and its ability to bind to ligands such as laminin, agrin, neurexin and perlecan is vastly abolished in all of these conditions [24]. Further, the CNS phenotype in *Large*^{myd} mice is almost identical to the phenotype in mice with a brain-specific DG gene deletion indicating that the loss of α -DG-ligand binding accounts for the defects in CNS development [25].

Using the *Large*^{myd} mouse as an *in vivo* system, we show here that NMJ maintenance is complexly compromised in *Large*^{myd} mice. Presynaptic nerve terminal differentiation is severely disrupted leading to sprouting and exuberant nerve growth. We show that AChRs are present at the NMJs of *Large*^{myd} muscle fibers but that the binding affinity to the venom toxin α -bungarotoxin is greatly decreased in a disease-course dependent manner. In addition, AChRs surface expression in cultured *Large*^{myd} muscle cells is diminished. Further, the extent of *O*-linked glycosylation is reduced at NMJs of *Large*^{myd} mice. Taken together, our data indicate that presynaptic and postsynaptic NMJ differentiation are highly dependent on correct glycosylation of the macromolecular endowment by *Large*.

2. Materials and Methods

2.1 Animals

Colonies of mdx (C57BL10 background) and *Large^{myd}* (C57BL6 background) mice were kept at the Animal Breeding Facilities of the Medical University of Vienna (Himberg). Healthy litter mates were used as wild-type control animals. New-born mice were between P3 and P5, adult mice were used between P30 and P90.

2.2. Antibodies and reagents

The rabbit polyclonal anti-AChE antibodies were a gift from Dr. T. Rosenberry (Mayo Clinic). Antibodies against rat agrin were provided by Dr. M. Ruegg (Biozentrum Basel). Rabbit anti- α -DG was a generous gift from Dr. S. Kroeger (Ludwig-Maximilians-Universität, Munich) and a polyclonal rabbit serum against α -syntrophin was kindly provided by Dr. S. Froehner (University of Washington). The rabbit antibodies against anti-ErbB4, anti-rapsyn and anti-MuSK were described previously [26-28]. The following antibodies were purchased from commercial sources: anti-neurofilament (Chemicon), anti-synaptophysin (Zymed), monoclonal anti-utrophin (Novocastra Laboratories), monoclonal anti-AChR α (Sigma) and anti-actin (Sigma). Alexa 594-conjugated α -bungarotoxin (BGT), Oregon green-conjugated α -BGT and biotin-conjugated α -BGT were obtained from Molecular Probes. Alexa 488-conjugated goat anti-rabbit and anti-mouse IgG and Texas Red-conjugated Streptavidin were purchased from Invitrogen and Amersham Bioscience, respectively. Biotinylated VVA was purchased from Vector Laboratories and recombinant PNGase F was obtained from Roche Diagnostics.

2.3 Immunohistochemistry

Diaphragm muscles were stained as described previously [26]. Briefly, dissected and fixed muscles were stained overnight at 4°C with antibodies against neurofilament and synaptophysin in 2% BSA, PBS, 0.5% Triton X-100, washed three times for 20 minutes in PBS, 0.5% Triton X-100 and incubated overnight at 4°C with Alexa 488-conjugated goat anti-rabbit IgG and Alexa-594-conjugated α -BGT. The muscles were washed twice for 20 minutes in PBS, 0.5% Triton X-100, twice in PBS and postfixed in 1% paraformaldehyde (PFA), rinsed in PBS and flat mounted in Vectashield (Vector Labs). Mouse leg (*quadriceps femoris*, *QF* and *tibialis anterior*, *TA*) muscle cryosections, stored at -80°C, were thawed at room temperature, fixed with 1% PFA for 5 minutes and rinsed in PBS. Muscle sections were incubated with 0.1M glycine in PBS for 10 minutes, permeabilized with 0.1% Triton/PBS for 10 minutes and blocked in 2% BSA in 0.1% Triton/PBS for 30 minutes. The sections were incubated with primary antibodies overnight at 4°C, washed three times with 0.1% Triton/PBS and incubated at room temperature for 2 hours with Alexa 488-conjugated secondary antibodies and Alexa 594-conjugated α -BGT to label synaptic AChRs. Finally, the muscle sections were washed three times with PBS, post-fixed in 1% PFA for 5 minutes and mounted in Vectashield. The stained whole-mount diaphragm muscles and sections were captured using an Olympus FluoView confocal laser scanning microscope. AChR signals (AChR staining normalized against α -BGT staining) were quantified using ImageJ software (NIH). Quantification of postsynaptic proteins was performed using ImageJ software by quantifying the mean grey values of identical regions-of-interests within the images stained with α -BGT and the antibodies against appropriate synaptic proteins. At least 10 NMJs were quantified for each synaptic protein.

2.4 Lectin staining of cryosections

Cryosections of QF and TA muscles were incubated with lectins diluted in 10mM HEPES, pH 7.5, 0.15 M NaCl at room temperature for one hour, washed twice for 5 minutes with

100 mM Tris, pH 7.5, 100 mM NaCl (TBS) and incubated with Texas Red-conjugated streptavidin and Oregon green-conjugated α -BGT at room temperature for one hour. Sections were washed twice with TBS and mounted in Vectashield.

In the case of enzyme treatment, sections were pretreated with PNGase F (5 units) in 50mM Tris-Cl, pH 7.0 for one hour or overnight at 37°C. For glycolipid extractions, sections were treated with methanol/chloroform (1:1) for 45 minutes at -80°C before lectin staining.

2.5 Staining of AChE activity

AChE activity was visualized according to protocols developed by Koelle and Friedenwald [29].

2.6 AChR pull-down and Western blotting

Muscle tissues were homogenized in lysis buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 50 mM sodium flouride, 1mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin and 1 μ M PMSF). After addition of an equal volume of lysis buffer containing 2% Triton X-100, the lysates were incubated at 4°C for 30 minutes and pre-cleared by centrifugation (20 minutes at 15,000 g). Protein concentration was determined using Rotiquant (Roth) and equal amounts of protein were incubated with biotin-conjugated α -BGT at 4°C overnight, followed by incubation with streptavidin agarose (Novagen). Bound proteins were eluted in SDS-PAGE sample buffer and analyzed by Western blotting with anti-AChR α antibody. Total cell lysates were analyzed by Western blotting with anti-actin and anti-AChR α antibodies. Immuno blots were scanned with a Fluor-S MultiImager (Bio-Rad Laboratories) and quantifications were performed using the Quantity One software (Bio-Rad Laboratories). Muscle cells were incubated with biotin-conjugated α -BGT in DMEM at 37°C for one hour. Cells were washed three times with PBS and lysed in 1% triton, 150 mM NaCl, 20 mM HEPES, pH 7.4, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 50 mM sodium flouride, 1mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin and 1 μ M PMSF. Pre-cleared lysates were used for AChR affinity purification and analysis by Western blotting as described above.

2.7 Cell culture

Lower leg muscles from 1 month old mice was dissected free from bone, tissue was dissociated in 2% trypsin (Sigma) and 0.01% DNase (Sigma) in PBS and cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine, 4.5 mg/ml glucose, 10% fetal bovine serum, 10% horse serum, 0.5% chick embryo extract and 100 mg/ml penicillin/streptomycin. Cells were pre-plated on a Petri dish for 20 minutes at 37°C to preferentially deplete connective tissue cells, and the less adherent cells in the supernatant were transferred to a Matrigel-coated tissue culture dish. Myoblasts were maintained for several days at 37°C. To induce myotube formation cells were re-plated at high density on 35 mm Matrigel-coated tissue plates and switched to low serum differentiation media composed of DMEM, 4.5 mg/ml glucose, 100 mg/ml penicillin/streptomycin and 10% horse serum. Soluble neural (A4B8) agrin was prepared from HEK 293T cells as described previously [27].

2.8 AChR clustering and immunocytochemistry

To induce AChR clustering, myotubes were stimulated with agrin overnight. To label surface AChRs, cells were incubated for 1 hour with Alexa 594-conjugated α -BGT, washed three times for 5 minutes with PBS, fixed in 1% PFA for 10 minutes and rinsed in PBS. Following an incubation in 0.1M glycine in PBS for 10 minutes, cells were permeabilized

with 0.2% NP-40/PBS for 5 minutes, blocked in 2% BSA, 0.2% NP-40/PBS for 30 minutes and stained with anti-AChR α antibody in blocking solution at room temperature for two hours. Cells were washed with PBS three times for 20 minutes, incubated with secondary Alexa 488 goat anti-mouse IgG for one hour, washed three times with PBS, postfixed in 1% PFA and mounted under coverslips. To label total AChRs, cells were fixed and permeabilized as described above. After staining with primary antibody, cells were washed with PBS and incubated with Alexa 488-conjugated goat anti-mouse IgG and Alexa 594-conjugated α -BGT for one hour. Subsequent treatment was as described above. The cells were viewed with a Zeiss Axioplan using optics selective for Alexa 594 and Alexa 488 and pictures were captured with a digital camera (F-View Soft Imaging System). Images were analyzed using ImageJ. AChR signals not co-localizing with surface α -BGT staining were quantified by measuring the calibrated mean grey values within a defined region-of-interest. AChR clusters were identified by visual screening for BGT staining and their size was determined by measuring the cluster area. AChR levels and cluster size in wild-type cells were set at 100%.

3. Results

3.1 Compromised NMJ development in *Large^{myd}* mice

To learn about NMJ development in glycosylation-deficient *Large^{myd}* mice we initially visualized AChE activity in whole-mount diaphragm muscles from adult *Large^{myd}* and control mice according to Koelle and Friedenwald [29]. As shown in Fig. 1A AChE is present in the middle of the muscle in wild-type mice but widely spread in *Large^{myd}* mice. To extend this finding, we stained whole-mount diaphragm muscles with antibodies against neurofilament and synaptophysin to label axons and nerve terminals, respectively. Already at low magnification we observed that the endplate zone of *Large^{myd}* mice was greatly increased and that nerve terminals appeared to grow long distances from the main axon (Fig. 1B). In contrast, wild-type mice revealed a characteristic narrow endplate zone with nerve terminals differentiating close to the main axon, as soon as they reached the muscle. At higher magnification nerve terminals in *Large^{myd}* mice appeared fragmented and less complex than wild-type mice (Fig. 1C). Further, we found that a significant number of nerve terminals produced sprouts that grew beyond the synaptic sites. In some instances these sprouts innervated the muscle at a second site and formed an additional NMJ on either the same or the neighboring muscle fiber. Additionally, many axons had an atrophic appearance (thin with varicosities) and undifferentiated nerve terminals that appeared to have lost contact to the muscle and that looked like retracting axons were readily visible in *Large^{myd}* mice. The complex pathology of the presynaptic NMJ apparatus was not confined to the diaphragm but was also visible in all other muscles examined including the *tibialis anterior*, *extensor digitorum longus* (EDL), *gastrocnemius* muscle and the *adductor magnus femoris* muscle (data not shown). We next wanted to know whether these presynaptic defects are also present during early development when muscle dystrophy has not yet developed. We stained whole-mount diaphragm muscle from newborn mice. In these young animals, the endplate zone remains within a narrow band in the middle of the muscle and nerve terminals differentiate at a discrete site on the muscle membrane (data not shown). However, as shown in Fig. 1D, axon sprouting is already apparent at this stage since a substantial number of synapses present nerve endings that grow beyond the site of innervation.

Differentiation at the NMJ occurs in parallel at pre- and at postsynaptic sites and are highly reciprocally dependent: as presynaptic nerve terminals differentiate, AChRs become highly concentrated at postsynaptic sites. To study postsynaptic differentiation we stained whole-mount diaphragm muscle from adult *Large^{myd}* and control mice with Alexa 594-conjugated α -bungarotoxin (BGT), which binds tightly and specifically to AChRs. The presynaptic apparatus was visualized with antibodies against neurofilament and synaptophysin. As

shown in Fig. 2A, postsynaptic differentiation, like presynaptic differentiation, is structurally severely disrupted in *Large^{myd}* mice. α -BGT staining is strongly reduced although AChRs still co-localize with nerve terminals. The same is also true for *gastrocnemius*, EDL and *m. adductus magnus* (data not shown). In contrast, the level of α -BGT staining at NMJs is indistinguishable between prepathologic muscles of newborn *Large^{myd}* mice and wild-type littermates (Fig. 2B). Thus, postsynaptic differentiation appears normal during early development.

At this point we speculated that the pre- as well as postsynaptic NMJ defects in older *Large^{myd}* mice could be caused by the dystrophic changes in the muscle tissue rather than by defective glycosylation. Therefore, we stained whole-mount diaphragm muscle from mdx mice, a murine model for Duchenne muscular dystrophy caused by a mutation in the dystrophin gene [30]. Like *Large^{myd}* mice, the mdx pathology is also characterized by progressive dystrophic changes in striated muscles, most prominently the diaphragm. NMJs in mdx muscle have a normal appearance (Fig. 2A). Nerve terminals appropriately differentiate and form characteristic pretzel-like structures apart from a mild fragmentation of NMJ structures, which has been reported previously [31]. Also, levels of AChRs are normal as detected with α -BGT, not only on whole-mount diaphragm muscles but also on cross sections from a variety of other skeletal muscles from mdx mice (data not shown). Therefore, we conclude that muscular dystrophy *per se* does not cause the NMJ defects seen in *Large^{myd}* mice but that a glycosylation-dependent effect is more probable to be the causative mechanism. This model is further supported by the finding that NMJs in *Large^{myd}* mice are also functionally defect (supplementary Fig. 1). Analysis of the jitter revealed an increased variability of neuromuscular transmission time between a series of recordings. Quantification of the mean consecutive difference (MCD) showed a jitter increase of about 60% between wild-type and *Large^{myd}* mice whereas the latency of occurring postsynaptic potentials is slightly but not significantly increased in *Large^{myd}* mice compared to wild-type mice indicating that the machinery for conducting an action potential along the axon and for neurotransmission at the nerve terminal is intact. In addition, muscle action potentials (MAPs) are of similar sized amplitude in wild-type and mutant mice pointing to the fact that despite the observed progressive muscle degeneration the overall response of muscle fibers in *Large^{myd}* mice is not significantly compromised.

3.2 Localization of synaptic proteins in *Large^{myd}* mice

Besides the accumulation of AChRs, NMJ formation is characterized by the concentration of a variety of proteins to synaptic sites. These proteins are important for NMJ function as well as NMJ formation and maintenance. We stained muscle sections for proteins known to be clustered at NMJs (Fig. 3). Agrin, MuSK, and rapsyn are mediators of AChR clustering and crucial for NMJ formation [32-34]. All three proteins are present at NMJs of *Large^{myd}* mice. Further, ErbB4, a receptor tyrosine kinase, which is able to induce AChR transcription in a neuregulin-dependent manner, is also localized at NMJs in *Large^{myd}* mice [28]. Similarly, acetylcholine esterase (AChE) was found at NMJs at levels comparable to wild-type. To determine the level of protein expression, we performed a detailed quantitative analysis (Table 1). Consistent with the data shown in Figure 2, α -BGT staining is significantly reduced in *Large^{myd}* mice compared to wild-type mice. The levels of agrin, rapsyn, ErbB4 and AChE are slightly but not significantly reduced at NMJs of *Large^{myd}* mice (about 80% of wild-type). In contrast, MuSK protein at NMJs of *Large^{myd}* mice is decreased to about 60% of wild-type NMJs.

α -DG, which is currently the only known target of Large, is expressed throughout the muscle membrane but is also highly enriched at the NMJ. Its expression at the synaptic sites is essential for NMJ maintenance and stability [18]. We found that α -DG expression at the muscle membrane is significantly reduced but that α -DG is still localized at NMJs of

Large^{myd} mice (Fig. 3 and Table 1). The family of syntrophin proteins is part of the DGC, whereby syntrophin $\beta 2$ is specifically enriched at the NMJ [35]. Using pan-syntrophin antibodies we found that syntrophin proteins are expressed at similar levels at the synaptic muscle membrane in wild-type and *Large^{myd}* mice. In contrast, the dystrophin-homologue utrophin is reduced at NMJs in *Large^{myd}* mice (Fig. 3 and Table 1). These results indicate that the expression or localization of the NMJ-specific DGC members α -DG and utrophin is compromised in *Large^{myd}* muscle.

3.3 Reduction of α -BGT binding to AChRs in *Large^{myd}* mice

As shown in Figure 2, α -BGT labeling of AChRs suggests a reduced number of AChRs at NMJs of *Large^{myd}* mice. Since the binding site for α -BGT lies within the N-terminal part of the AChR α subunit and is close to a *N*-glycosylation site we were interested to find out whether there are indeed fewer AChRs present or whether α -BGT binding is reduced due to altered AChR glycosylation [36, 37]. We stained muscle sections with antibodies against the AChR α subunit and α -BGT. As shown in Fig. 4A, AChR α staining is slightly but not significantly reduced in *Large^{myd}* mice (79.3% $\pm$ 7.2%) compared to wild-type mice (100% $\pm$ 4.5%). This is in contrast to the α -BGT binding, since α -BGT staining is strongly reduced in *Large^{myd}* muscle (52.0% $\pm$ 3.5%) compared to wild-type muscle (100% $\pm$ 9.1%). To follow up on this observation, we prepared lysates from muscle tissue, affinity-purified AChRs with α -BGT and separated the proteins by SDS-PAGE. The ability of α -BGT to bind to AChRs was determined by Western blotting with antibodies against AChR α (Fig. 4B). In newborn mice, α -BGT binds similarly well to AChRs in *Large^{myd}* muscle compared to wild-type muscle. However, in mice older than four weeks the binding affinity of α -BGT to AChRs is severely reduced in *Large^{myd}* muscle compared to wild-type muscle. The binding affinity of α -BGT to AChRs is not affected in mdx mice. Using the biochemical isolation of AChR with α -BGT we see a more pronounced effect as with immunohistological methods. A reason might be that in the biochemical assay we look at whole muscle lysates, which might enhance the effect compared to the muscle section staining where we specifically compare synaptic AChRs. When we analyzed total AChR protein levels in muscle we found that AChR α is slightly increased in *Large^{myd}* mice compared to wild-type mice, possibly a consequence of altered innervation as seen in Figure 1 (Fig. 4C). However, this indicates that diminished AChR expression is not responsible for the difference in the amount of AChR α brought down by α -BGT. Thus, reduced α -BGT binding to AChRs in *Large^{myd}* muscle implies that AChR glycosylation might be altered in *Large*-deficient mice.

3.4 Altered glycoepitope endowment at NMJs of *Large^{myd}* mice

We were interested to find out whether a mutated *Large* glycosyltransferase causes a differential glycosylation pattern specifically at NMJs. For that reason and also due to its previously described strong specificity towards the NMJ we stained muscle sections of *Large^{myd}* and wild-type mice with the lectin *Vicia villosa* (VVA) [38]. AChRs were co-labeled with α -BGT to mark NMJs. Our results confirmed a prevalence of VVA-binding sites within the synaptic region, whereas sarcolemmal binding was barely detectable (Fig. 5A). As previously shown in Figure 2 and 4, α -BGT staining of the NMJs in *Large^{myd}* muscles was greatly reduced and partially missing, whereas α -BGT clearly stained NMJs of wild-type mice. In contrast, the intensity of VVA staining on *Large^{myd}* NMJs was similar to wild-type NMJs, although the NMJs appeared thinner and more segmented as predicted from their altered morphology (Fig. 2).

To specify the detected glycan structures in more detail, we digested the sections with different combinations of enzymes prior to probing the digested sections with VVA and α -BGT. Specifically the following treatments were performed: (i) with neuraminidase alone in

order to test whether some VVA epitopes were cryptic due to masking with sialic acid, (ii) with chicken α -GalNAcase alone to test whether the interactions with VVA were due to the Tn-antigen (GalNAc α -linked to serine or threonine) and (iii) with a cocktail of neuraminidase, β 1,3-, β 1,4- and α -galactosidases, hexosaminidase and α -GalNAcase, which should remove most 'mucin'-type *O*-linked structures and trim commonly-known complex *N*-glycans down to the trimannosyl core. However, none of these treatments resulted in an obvious alteration in VVA staining, other than that α -GalNAcase removed all extrasynaptic staining (data not shown).

Since the β -GalNAc containing CT-antigen, which strongly interacts with VVA, has already been described as highly resistant to sialidase and hexosaminidase treatment, this result provided further indications that we were detecting a GalNAc β 1,4[Neu5Ac α 2,3]Gal sequence [39-41]. GalNAc β 1,4[Neu5Ac α 2,3]Gal groups can be found as terminal sequences of glycolipids (e.g., GM2, GD2), *N*- and *O*-glycans. Thus, we extracted the glycolipids with chloroform/methanol or digested muscle sections from wild-type, *Large^{myd}* and dystrophin-deficient mdx mice with PNGaseF, which removes all *N*-glycans from mammalian peptide chains (Fig. 5B). Removal of glycolipids only led to a minor decrease of intensity in all genotypes. Interestingly however, after removal of the *N*-glycans, we observed a nearly complete loss of staining of the NMJs in *Large^{myd}* mice. In contrast, NMJs were still clearly visible in wild-type and mdx muscle (Fig. 5B). This suggests that NMJs in *Large^{myd}* muscles are lacking the CT-antigen on *O*-glycosidically linked carbohydrate moieties, whilst retaining it on *N*-glycans, whereas VVA-specific *O*-glycans and *N*-glycans are present in wild-type and mdx mice.

3.5 Reduced surface expression of AChRs in muscle cells prepared from *Large^{myd}* muscle

It has previously been shown that DG is important for the formation of stable and condensed synaptic-like AChR clusters [18]. These results were obtained in myotubes from ES cells that lack α - and β -DG expression. To test whether an altered α -DG glycosylation might affect AChR cluster formation in a similar way we isolated primary myoblasts from *Large^{myd}* and wild-type mice and differentiated them into myotubes. *Large^{myd}* and wild-type myoblasts formed myotubes of equivalent size but primary cells isolated from *Large^{myd}* muscle contained fewer myoblasts and more other cell types, especially fibroblasts, than wild-type cultures (data not shown). Thus, differentiated *Large^{myd}* primary cultures contained fewer myotubes in total compared to wild-type cultures. Myotubes were treated with soluble agrin and stained with anti-AChR α antibody and α -BGT. Surface AChRs were labeled with α -BGT with subsequent staining of total (surface and intracellular) AChRs with anti-AChR α antibody. Wild-type and *Large^{myd}* myotubes are able to form AChR clusters in response to agrin. However, AChR clusters on *Large^{myd}* cells are bigger in size and AChRs are arranged diffusely and less condensed compared to wild-type AChR clusters (Fig. 6A). In addition, a considerable amount of AChRs remains intracellularly in *Large^{myd}* myotubes (compare merged AChR/BGT images of wild-type and *Large^{myd}* cells and Fig. 6C). When surface and intracellular AChRs are stained with both, anti-AChR α antibody and α -BGT, AChR α and α -BGT signals match exactly indicating that antibodies against AChR α and α -BGT recognize surface and intracellular AChR to a similar degree (Fig. 6B). When, surface AChRs were isolated by a α -BGT affinity purification we found that fewer AChRs are contained in the surface fraction in *Large^{myd}* muscle cells compared to wild-type muscle cells. Vice versa, more AChRs are present in the cytosolic fraction in *Large^{myd}* muscle cells compared to wild-type muscle cells (Fig. 6D and 6E). These data fit well with the increase in cytosolic AChRs found in Figure 6A and 6C. Therefore, the glycosylation defect in *Large^{myd}* muscle cells appears to reduce the amount of AChRs on the cell surface either by interfering with AChR assembly or AChR transport. Further, the appearance of AChR clusters in *Large^{myd}* myotubes in response to agrin is reminiscent of

agrin-induced AChR clusters in DG^{-/-} myotubes [18]. Clusters are less densely packed with AChRs and cover a larger area on the myotube surface (Fig. 6A and 6F). These results suggest that α -DG glycosylation by Large plays a role during AChR cluster maintenance.

4. Discussion

Large^{myd} mice display several developmental defects like myodystrophy, cardiomyopathy, ocular abnormalities and aberrant nerve conduction [20, 21, 42, 43]. Mutations in *Large* result in a hypoglycosylation of α -DG, which is causally associated with the defects seen in *Large^{myd}* mice [20, 21]. In this study we demonstrate aberrant presynaptic NMJ differentiation associated with altered postsynaptic DGC composition. Furthermore, α -BGT binding to muscle AChRs is diminished and AChR surface distribution is affected in muscle cells isolated from *Large^{myd}* muscle. These results together with the finding that O-linked glycosylation at NMJs is compromised implicate Large-dependent glycosylation events in NMJ development.

4.1 Structural alterations of pre- and postsynaptic organizations in *Large^{myd}* mice

Consistent with previous studies we have found that NMJs of *Large^{myd}* mice are structurally disrupted [43, 44]. In particular, we show a dramatic presynaptic nerve terminal defect associated with postsynaptic alterations (Fig. 1 and 2). This nerve defect is weakly detectable in new-born animals and becomes strikingly evident by P30. In contrast, *mdx* mice reveal only subtle defects in NMJ structure and normal presynaptic nerve growth. This suggests that the defects are not an indirect consequence of muscle dystrophy seen in *Large^{myd}* mice but rather a glycosylation-dependent effect as a result of lacking Large activity. Our findings are in good agreement with EM data from FCMD patients and *Large^{myd}* mice that demonstrated ultrastructural alterations like abnormal nerve endings and fewer synaptic folds [44]. In addition, functional analysis of neurotransmission in *Large^{myd}* mice using single fiber electromyography revealed that the activity of NMJs is aberrant (supplementary Fig. 1). Similar changes in neuromuscular transmission, which are caused by pre- and postsynaptic alterations have been reported in myasthenia gravis, during reinnervation and botulinum intoxication [45]. Taken together, these data are consistent with previous findings that showed an association between structural abnormalities and impaired neurotransmission [46, 47].

Whether the nerve phenotype is caused by a defect intrinsic to the nerve or by a postsynaptic defect is unclear. Current data however support a model whereby hypoglycosylation of α -DG in *Large^{myd}* muscle might lead to a disruption of the DGC at the NMJ causing nerve sprouting due to changes in the extracellular matrix (ECM). An instructive role of the ECM in nerve sprouting is further supported by the findings that nerve terminals at NMJs of chimeric mice lacking DG are larger and fragmented, that laminin can substitute for agrin in stimulating AChR clustering and that the ECM can promote the assembly of a pretzel-like postsynaptic apparatus *in vitro* without nerve-derived signals [17, 48, 49]. Interestingly it has been shown that hypoglycosylated α -DG is unable to bind agrin, which has recently also been implicated in NMJ stability [21, 50, 51]. The role of the agrin/ α -DG interaction is however unclear. Alternatively, altered glycosylation of α -DG in Schwann cells may contribute to the presynaptic defect seen in *Large^{myd}* mice. It has been reported that myelination of peripheral nerves is abnormal and that Schwann cell-axon relations are disrupted in *Large^{myd}* mice [43]. This is consistent with observations in mice with a Schwann cell-specific deletion of DG [52]. However, a defect in Schwann cells would rather induce nerve atrophy and degeneration than nerve sprouting as observed in *Large^{myd}* mice [53, 54].

4.2 Characterization of the molecular endowment at NMJs of *Large^{myd}* mice

The NMJ is characterized molecularly by the enrichment of specific proteins to the pre- and postsynaptic membrane. These proteins are involved in NMJ formation like MuSK and rapsyn, or in NMJ function and stability like syntrophin and α -DG [13, 18, 32, 33]. Previously, Levedakou and colleagues report a reduced expression of agrin, MuSK, rapsyn and AChE at NMJs of *Large* mutant mice (*myd* and *enr*) [43]. This is only in part consistent with our data since we detect a slight but non-significant reduction of protein expression for rapsyn, agrin, ErbB4 and AChE, which corresponds to the reduction of synaptic AChR expression and is probably correlated with the disrupted morphology of *Large^{myd}* NMJs (Fig. 3 and Table1). We are currently unable to explain the difference to the published data but the use of different antibodies might play a role. The normal levels of AChE at NMJs of *Large^{myd}* mice is somewhat surprising since AChE is absent from synaptic sites in *DG^{-/-}* myotubes and in *DG*-deficient chimeric mice [18]. Our results therefore demonstrate that α -DG glycosylation is not required for localizing AChE at NMJs. As mentioned above, hypoglycosylated α -DG is unable to bind agrin. Since we find agrin still localized at NMJs in *Large^{myd}* mice we further conclude that the localization of agrin is also not dependent on α -DG glycosylation. Consistent with the previous report, MuSK protein is significantly diminished at NMJs in *Large^{myd}* mice indicating that the expression of MuSK is more susceptible to changes in the NMJ morphology [43]. In addition, we find that the synaptic enrichment of the DGC proteins, α -DG and utrophin is reduced in *Large^{myd}* mice. A possible explanation could be the hypoglycosylation of α -DG, which might lead to a disruption of the utrophin-based DGC at the NMJ. Alternatively, the lack of α -DG and utrophin accumulation could be a result of the structural alterations of the postsynaptic muscle membrane in *Large^{myd}* mice, which is characterized by fewer postsynaptic folds [44]. Our finding that initial NMJ formation in young *Large^{myd}* mice is normal, is consistent with previous studies showing that α -DG is not involved in NMJ formation, which depends on agrin/MuSK signaling but rather regulates NMJ maintenance and stability [18]. This is further sustained by the ability of *Large^{myd}* muscle cells to respond to agrin stimulation. AChR clusters are formed in *Large^{myd}* muscle cells in response to agrin. These clusters are however larger in area and AChRs are more dispersed and less dense than in wild-type muscle cells (Fig. 6). The appearance of these AChR clusters is similar to the appearance of AChR clusters in *DG^{-/-}* muscle cells indicating that a lack of α -DG glycosylation has a similar effect on AChR cluster formation as a deficiency in DG expression [18].

4.3 AChRs: a possible indirect target of *Large* activity

Using α -BGT to label AChRs we found that α -BGT binding to AChRs in *Large^{myd}* mice is greatly reduced. Our initial explanation that fewer AChRs are expressed at NMJs in *Large^{myd}* muscle was contradicted by immunohistological and biochemical experiments demonstrating similar levels of AChRs at NMJs in wild-type muscle and *Large^{myd}* muscle (Fig. 4). Nicotinic AChRs consist of four different subunits, which are assembled into a $\alpha 2\beta\gamma\delta$ pentamer in foetal and $\alpha 2\beta\epsilon\delta$ in adult vertebrate muscle [55]. Binding of α -BGT was located to residues between positions 185 and 196 of the N-terminal domain of the α -subunit [37]. However, certain regions of γ or δ subunits have been shown to also contribute to α -BGT binding [56]. The five subunits contain a total of eight consensus sequences for *N*-glycosylation, whereby each subunit contains one glycan with a high mannose structure [57, 58]. The three remaining carbohydrate chains, located on the γ -subunit and δ -subunit, seem to be complex-type *N*-glycans [58]. It has been shown that a non-glycosylated form of the AChR α -subunit binds α -BGT with lower affinity [4, 5]. Furthermore, α -BGT binding is completely blocked when the *N*-glycosylation consensus site is mutated implicating glycosylation as critical event for α -BGT binding [59].

α -BGT binding site formation and AChR complex assembly represents a multi-step process. Subunits are assembled into a pentamer in the ER, subsequently folding of the complex into a functional receptor occurs and transport to and insertion into the plasma membrane follows. Posttranslational processing events, such as the formation of disulfide bonds and addition of *N*-linked glycans have been implicated in all of these steps [6, 7, 60, 61]. The reduced α -BGT binding in *Large^{myd}* mice suggests that altered glycosylation of AChRs either affects directly the binding affinity of α -BGT or interferes with correct AChR assembly, which would affect α -BGT binding site formation. Incorrect assembly of AChRs would further impinge on their transport to and insertion into the plasma membrane, which is supported by our finding that AChRs are found largely intracellularly in muscle cells isolated from *Large^{myd}* muscle (Fig. 6).

It appears unlikely that AChRs are a direct target of *Large* since AChRs lack *O*-linked glycans and oligomannosidic glycans of the type found on the α -subunit of AChRs are probably not targets for glycomodification. However, *Large* has been found to potentially modulate other glycosyltransferases [62], which in turn might be involved in the glycosylation of AChRs or of proteins, which affect AChR distribution.

4.4 O-linked glycosylation is compromised in *Large^{myd}* mice

We found that glycosylation of NMJs from *Large^{myd}* mice is significantly different to mdx and wild-type mice. Whereas NMJs from wild-type, but also dystrophic mdx muscle express VVA epitopes on *N*- and *O*-glycans, *O*-glycosidically linked VVA recognition sequences, either mucin type or *O*-mannosyl, are dramatically reduced at NMJs of *Large^{myd}* muscles (Fig. 5). Our working hypothesis is that VVA interacts with the β -GalNAc-containing CT antigen and that these carbohydrate moieties are missing from NMJs of *Large^{myd}* mice. Several lines of evidence support this: (1) Lectin studies have shown a high selectivity of VVA towards terminal GalNAc [41, 63-65]. (2) Digestion with α -GalNAcase, which removes the Tn antigen removed the extrasynaptic VVA staining from all genotypes, but did not reduce staining at the NMJs. (3) Enzyme treatments with various exo-glycosidases, which would remove all mucin-type *O*-glycans and cut down most usual types of complex *N*-linked sugars to the trimannosyl core, only led to minor changes. (4) Removal of *N*-glycans by PNGase F revealed a reduction of VVA staining in *Large^{myd}* muscle indicating that mucin type or *O*-mannosyl glycans are missing (Fig. 5B). One possibility is that altered glycosylation or hypoglycosylation results in an inability of CT GalNAc transferase to transfer GalNAc residue to its normal substrates. Thus, the decrease in VVA staining is hypothesized to be due to an absence of appropriate non-*N*-glycan substrates for the CT transferase rather than that *Large* itself generates CT epitopes.

Histochemistry studies with various lectins, amongst them VVA and anti-carbohydrate antibodies, have revealed that carbohydrate structures, expressing terminal β -linked GalNAc are specifically concentrated at the NMJ, whereas other glycans are equally distributed synaptically and extrasynaptically [2, 38, 66, 67]. Recently, indications have accumulated that α -DG is amongst the proteins that carry terminal β -GalNAc residues [66, 68]. α -DG possesses a large mucin-like domain with many serine or threonine residues, which are potential sites for *O*-glycosylation, which theoretically could be modified by the CT transferase [69]. Furthermore α -DG is known to be subject to *O*-mannosylation [70]. Although our results point to the fact that the NMJ defects seen in *Large^{myd}* mice are a consequence of α -DG hypoglycosylation, glycosylation of molecules, other than α -DG, could also be affected by the lack of *Large*. It remains therefore of particular interest to identify targets of *Large* glycosylation and how these proteins interact with other components, such as AChRs, of the NMJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Aberrant presynaptic nerve terminal differentiation in *Large^{myd}* mice. (A) Diaphragm muscles were stained according to Koelle and Friedenwald [29] to visualize AChE. AChE is widely distributed in *Large^{myd}* muscle compared to wild-type muscle. Bar, 100 μ m (B) Whole-mount diaphragm muscles were stained with anti-neurofilament and anti-synaptophysin antibodies. The endplate zone in *Large^{myd}* mice is enlarged and nerve terminals cover a large proportion of the muscle compared to wild-type mice. Bar, 100 μ m (C) NMJs in adult wild-type mice have a characteristic pretzel-like structure. In contrast NMJs in *Large^{myd}* mice are disrupted and axons sprout beyond the initial site of innervation (asterisks). Axon sprouts even innervate adjacent muscle fibers to form a second NMJ (arrowhead). Bar, 50 μ m (D) New-born *Large^{myd}* muscle show plaque-shaped nerve terminals similar to wild-type muscle. However, axon sprouting occurs already at these early immature NMJs (asterisks). Bar, 20 μ m

Fig. 2.

Compromised pre- and postsynaptic differentiation at NMJs in *Large^{myd}* mice. Diaphragm muscles were stained with antibodies against neurofilament (NF) and synaptophysin (SYN) and with α -BGT. (A) In adult wild-type and mdx mice nerve terminals differentiate and AChRs concentrate postsynaptically at the site of nerve innervation. NMJs in *Large^{myd}* mice are simplified and AChR accumulation is barely detectable. (B) AChR clustering is evident in new-born wild-type and *Large^{myd}* muscle. Bar, 20 μ m.

Fig. 3.

Immunohistochemical analysis of synaptic proteins in muscle of wild-type and *Large^{myd}* mice. Muscle sections were stained with antibodies against agrin, MuSK, rapsyn, ErbB4, AChE, α -DG, syntrophin (Syntr) and utrophin (UTR) and co-labeled with α -BGT. Agrin, MuSK, rapsyn, ErbB4 as well as AChE are present at NMJs in wild-type and *Large^{myd}* mice. Proteins of the DGC complex like α -DG and utrophin are, however, decreased at NMJs in *Large^{myd}* mice compared to wild-type muscle. Bar, 20 μ m.

Fig. 4. α -BGT binding to AChRs in *Large^{myd}* mice. (A) Muscle sections of wild-type, *Large^{myd}* and mdx mice were co-stained with antibodies against AChR α and with α -BGT. Binding of the anti-AChR α antibody is similar at wild-type, mdx and *Large^{myd}* NMJs. In contrast binding of α -BGT is greatly reduced in *Large^{myd}* NMJs compared to wild-type and mdx NMJs. Bar, 15 μ m. (B) AChRs were affinity-purified from muscle lysates of wild-type, *Large^{myd}* and mdx new-born (P3) and adult (P90) mice using α -BGT. Purified AChR α was detected by Western blotting. Protein loading was analyzed by Western blotting with anti-actin. In contrast to newborn muscle, the binding activity of α -BGT to AChRs in adult *Large^{myd}* muscle is reduced and less AChRs are isolated compared to wild-type and mdx

muscle. (C) Total protein lysates from diaphragm (DM) and quadriceps (QF) muscles from wild-type and *Large^{myd}* mice were analyzed by Western blotting using antibodies against AChR α and actin. (D) A quantification of several Western blot experiments (n=3) revealed that AChR α is expressed at a similar level in wild-type and *Large^{myd}* muscles.

Fig. 5. VVA staining of NMJs in wild-type, *Large^{myd}* and mdx mice. (A) Muscle sections were stained with VVA and α-BGT. VVA is specifically enriched at NMJs in wild-type and *Large^{myd}* mice. (B) Untreated (UT), glycolipid extracted (Chloro/MeOH) and PNGase F treated muscle sections were stained with VVA. Removal of N-glycans with PNGase F abolishes VVA binding to NMJs in *Large^{myd}* mice. Wild-type and mdx NMJs are unaffected by PNGase F treatment. Bar, 20 μm.

Fig. 6.

Agrin-induced AChR cluster formation in cultured muscle cells from wild-type and *Large^{myd}* mice. Primary myoblasts were isolated from leg muscle tissue, differentiated into myotubes and stimulated with agrin. (A) Surface AChRs were labeled with α -BGT whereas total (surface and intracellular) AChRs were stained with antibodies against AChR α . AChR clusters are induced by agrin in wild-type and *Large^{myd}* myotubes. However, AChR clusters in *Large^{myd}* muscle cells are enlarged and diffusely distributed. In addition, a substantial amount of AChRs are contained within intracellular compartments in *Large^{myd}* myotubes. (B) Muscle cells were permeabilized and total AChRs were co-stained with anti-AChR α and α -BGT. Merged images show an exact co-localization of anti-AChR α and α -BGT staining in wild-type and *Large^{myd}* myotubes. (C) Cytoplasmic AChR α staining within

myotubes was quantified. Muscle cells derived from *Large^{myd}* mice show an increase in intracellular AChR α expression. (D) Surface AChR were isolated using affinity purification via biotin-conjugated α -BGT. Western blot analysis revealed that less AChR α is contained within the surface fraction in *Large^{myd}* cells compared to wild-type cells. AChR α expression in total lysates shows that less muscle cells were isolated from *Large^{myd}* muscle than from wild-type muscle. (E) AChR α surface expression versus cytoplasmic expression was quantified. *Large^{myd}* cells express less AChR on the surface than wild-type cells. (F) The AChR cluster size was quantified in wild-type and *Large^{myd}* muscle cells treated with agrin. AChR clusters are enlarged in *Large^{myd}* cells compared to wild-type cells.

Table 1

Quantitative analysis of synaptic protein expression at NMJs of Large^{myd} mice. Mean grey values of α -BGT and antibody staining was quantified using ImageJ software. The mean absolute values including the standard error are shown. α -BGT, MuSK, α -DG and utrophin (UTR) are significantly reduced at NMJs of Large^{myd} mice compared to wild-type mice (*P*-values shown in bold)

	BGT	agrin	BGT	MuSK	BGT	Rapsyn	BGT	Erbb4	BGT	AChE	BGT	α -DG	BGT	Syn	BGT	UTR
WT	105.7 +/-12.8	178.4 +/-9.8	99.7 +/-8.2	133.1 +/-12.0	123.2 +/-12.1	103.6 +/-13.1	131.5 +/-8.7	123.5 +/-12.7	174.9 +/-7.9	243.5 +/-4.7	178.8 +/-9.8	125.9 +/-6.2	141.8 +/-7.9	199.8 +/-8.0	161.05 +/-8.3	136.1 +/-9.7
MYD	49.04 +/-8.5	131.5 +/-22.9	34.96 +/-6.2	68.8 +/-12.0	68.9 +/-11.9	78.7 +/-15.5	84.4 +/-9.7	111.5 +/-12.4	124.8 +/-13.3	230.6 +/-7.2	116.95 +/-16.1	89.2 +/-8.1	93.6 +/-9.1	175.9 +/-7.2	95.9 +/-10.3	90.5 +/-7.6
P	0.0018 **	0.294	0.0003 ***	0.0028 ***	0.0079 **	0.132	0.0037 **	0.397	0.026 **	0.224	0.012 **	0.0021 **	0.0017 **	0.053	0.0009 **	0.0029 **

*** $P < 0.05$ Mann-Whitney U -test.