DISTINCT ROLES OF SYNAPTIC βGalNAc TRANSFERASES, GALGT1 AND GALGT2, IN MUSCLE BIOLOGY

Dissertation

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By

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ABSTRACT

The vertebrate neuromuscular junction (NMJ), the synapse formed between the nerve terminal of the motor neuron and the postsynaptic membrane of the skeletal myofiber, remains the preeminent model synapse to study cellular and molecular aspects of synaptic development. All NMJs studied thusfar, including those in lampreys, axolotls, fish, frogs, chickens, dogs, cats, mice, rats, guinea pigs, hamsters, monkeys and humans, have highly concentrated synaptic localization of terminal β-linked N-acetyl-D-galactosamine (βGalNAc). Such synaptic βGalNAc structures can be made, in part, by Galgt1 and Galgt2, a family of two β1,4GaNAc transferases. While both of these enzymes synthesize synaptic β1,4GaNAc linkages to make Neu5Acα2,3[GalNAcβ1,4]Galβ-R glycans, such as those found at the NMJ, they do so on different classes of substrates, with Galgt1 able to glycosylate ganglioside-type glycolipids while Galgt2 glycosylates certain mucin-like glycoproteins. Here we show that both the Galgt2 and the Galgt1 proteins are concentrated in synaptic regions of skeletal myofibers and can contribute to the synthesis of synaptic βGalNAc glycans but that they do so in different ways, both with regard to expression and function.

Using mice containing genetic deletions of Galgt1, Galgt2, or both genes, we show that Galgt1 controls the expression of pre-synaptic βGalNAc structures on the
motor nerve terminal, while Galgt2 primarily controls the expression of postsynaptic ones in the muscle membrane. The Martin Lab has shown previously that overexpression of Galgt2 in skeletal muscle leads to the specific glycosylation of α dystroglycan, a member of the dystrophin-associated glycoprotein complex. Here, we show that loss of dystroglycan at the NMJ leads to loss of βGalNAc in the postsynaptic muscle membrane, suggesting α dystroglycan is required for postsynaptic βGalNAc localization. The Martin lab has also previously shown that Galgt2 overexpression in skeletal muscle induces the overexpression of synaptic dystroglycan-binding proteins and can, via such changes, inhibit the development of muscular dystrophy in multiple forms of the disease. Here, we show that overexpression of Galgt1, by contrast to Galgt2, does not allow for overexpression of extrasynaptic βGalNAc expression in the muscle membrane and instead leads to focal intracellular aggregates of βGalNAc expression that are associated with decreased muscle growth and increased muscle pathology. Thus, Galgt2 is clearly unique as a therapeutic target in muscular dystrophy.

Loss of Galgt2 in mice led to an age-dependent disorganization of postsynaptic nicotinic acetylcholine receptors (AChRs), the neurotransmitter receptors responsible for synaptic transmission at the NMJ. AChRs, along with acetylcholinesterase, but not other DAG proteins, were increased within endosomes in Galgt2-deficient muscles, suggesting a role for Galgt2 in AChR membrane stability as muscle age.

Using microarray studies of muscle regeneration data generated, we queried 1800 genes either directly responsible for glycosylation or encoding major glycoproteins
or lectins. Of these, Galgt1 was the gene most upregulated in response to muscle
damage induced by cardiotoxin. We confirmed an almost 30-fold increase in Galgt1
mRNA a day after cardiotoxin-induced muscle injury by qRT-PCR. FACS cell sorting points
to a high increase in Galgt1 expression in satellite cells within 1 day after muscle
regeneration begins, while CD31-positive endothelial cells and CD45-positive immune
cells show no increase. These data point to a role for Galgt1 on satellite cells, the stem
cells of skeletal muscle. Induction of muscle regeneration was delayed, both in kinetics
and extent, in mice lacking Galgt1, in response to cardiotoxin-induced injury.
Dystrophin-deficient mdx mice lacking Galgt1 also showed reduced muscle
regeneration, as evidenced by myofibers with smaller average diameters and also
reduced numbers of myofibers with central nuclei. In addition, in both experiments,
large aggregates of mononuclear cells could be seen that were indicative of a potential
in vivo migratory defect at sites of injury. These data support a role for Galgt1 in
skeletal muscle regeneration.
Dedicated to my Family.
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# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii
DEDICATION ........................................................................................................................... Error! Bookmark not defined.
ACKNOWLEDGEMENTS ....................................................................................................... vi
VITA .......................................................................................................................................... viii
LIST OF FIGURES ................................................................................................................ xii

CHAPTER 1. INTRODUCTION ................................................................................................. 1
   1.1. Introduction ................................................................................................................ 1
   1.2. Glycobiology of Neuromuscular Junction ............................................................... 2
   1.3. Role of Galgt2 in disease ........................................................................................... 10
   1.4. Neuromuscular Junction and its Development ......................................................... 11
   1.5. Skeletal Muscle Regeneration .................................................................................... 27

CHAPTER 2. Distinct contributions of Galgt1 and Galgt2 to synaptic carbohydrate expression and function at the mouse neuromuscular junction. ................................................. 34
   2.1. Introduction ................................................................................................................ 34
   2.2. Materials and Methods ............................................................................................. 36
   2.3. Results ......................................................................................................................... 40
   2.4. Discussion ................................................................................................................... 58

CHAPTER 3. Role of Galgt1 in skeletal muscle regeneration .................................................. 62
   3.1. Introduction ................................................................................................................ 62
   3.2. Materials and Methods ............................................................................................. 63
   3.3. Results ......................................................................................................................... 68
   3.4. Discussion ................................................................................................................... 106

Chapter 4. SUMMARY .......................................................................................................... 112
BIBLIOGRAPHY. .................................................................................................................. 115
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Biosynthesis of GM2 ganglioside</td>
</tr>
<tr>
<td>1.2</td>
<td>Biosynthesis of CT antigen</td>
</tr>
<tr>
<td>1.3</td>
<td>Developmental expression of synaptic extracellular matrix (ECM) proteins at the rodent neuromuscular junction</td>
</tr>
<tr>
<td>1.4</td>
<td>Synaptic extracellular matrix (ECM) protein agrin and its receptors at the neuromuscular junction</td>
</tr>
<tr>
<td>1.5</td>
<td>Synaptic ECM signaling in neuromuscular development</td>
</tr>
<tr>
<td>2.1</td>
<td>Galgt1 is expressed at the neuromuscular junction</td>
</tr>
<tr>
<td>2.2</td>
<td>Galgt1 and Galgt2 both contribute to expression of the CT carbohydrate at the neuromuscular junction</td>
</tr>
<tr>
<td>2.3</td>
<td>Deletion of dystroglycan expression in P3ProCreDag1loxP/loxP hindlimb muscles</td>
</tr>
<tr>
<td>2.4</td>
<td>Synaptic expression of the CT2 carbohydrate is altered in Dag1−/− muscle</td>
</tr>
<tr>
<td>2.5</td>
<td>Galgt1 overexpression in skeletal myofibers fails to induce membrane expression of CT carbohydrate and causes myopathic changes</td>
</tr>
<tr>
<td>2.6</td>
<td>Neuromuscular phenotype in Galgt2−/− muscle</td>
</tr>
</tbody>
</table>
2.7. Co-localization of intracellular AChR microaggregates with an endosomal marker in Galgt2−/− muscle. ................................................................. 56

3.1. Microarray data showing multi-fold increase in Galgt1 signal after cardiotoxin injection of skeletal muscle ................................................................. 69

3.2. Fold change in the expression of Galgt1 gene in WT muscle after cardiotoxin injection ................................................................................................. 71

3.3. Different stages of regeneration in WT Gastocnemius muscle injected intramuscularly with cardiotoxin (CTX) ................................................................. 74

3.4. Staining of the neuromuscular junction (NMJ) with GM1 and Galgt1 antibodies in WT and Galgt1−/− mice ........................................................................... 77

3.5. Galgt1 antibody staining in WT, mdx, Galgt1−/−, and Galgt1−/−-mdx muscle ....... 80

3.6. GM1 ganglioside staining in WT, mdx, Galgt1−/−, and Galgt1−/−-mdx muscle ...... 81

3.7. Different stages of regeneration in WT and Galgt1−/− ........................................ 84

3.8. Average myofiber diameter of WT and Galgt1−/− gastrocnemius at Day 14 after cardiotoxin (CTX) injury ................................................................. 85

3.9. Distribution of myofiber diameter of WT and Galgt1−/− gastrocnemius at Day 14 after cardiotoxin injury ................................................................. 86

3.10. Average myofiber diameter of WT and Galgt1−/− gastrocnemius at Day 28 after cardiotoxin injury ................................................................. 87

3.11. Distribution of myofiber diameter of WT and Galgt1−/− gastrocnemius at Day 28 after cardiotoxin injury ................................................................. 88
3.12. H&E staining of Gastrocnemius in young (6 weeks) and old (6 months) mdx and Galgt1-/mdx animals .................................................................................................................. 90

3.13. Average myofiber diameter of mdx and Galgt1-/mdx gastrocnemius at 6-months ........................................................................................................................................... 92

3.14. Distribution of myofiber diameter of mdx and Galgt1-/mdx gastrocnemius at 6-months. .............................................................................................................................................. 93

3.15. Average percentage central nuclei in mdx and Galgt1-/mdx gastrocnemius at 6-months ............................................................................................................................................ 94

3.16. Hematoxylin and eosin (H&E) staining of Gastrocnemius of WT, mdx, Galgt1-/ and Galgt1-/mdx mice at 6-months................................................................................................................................. 95

3.17. Creatine kinase (CK) assay at different time points of WT, Galgt1-/, mdx, mdx/Galgt1-/- ......................................................................................................................................................... 96

3.18. Fluorescent activated cell sorting (FACS) of A. Cardiotoxin injected WT muscles and B. Non-injected WT muscles ........................................................................................................................................ 99

3.19. Real-time quantification of mRNA extracted from the Sca1 sorting. .................. 101

3.20. Real-time quantification of mRNA extracted from the integrin α7 sorting....... 102

3.21. Double immunostaining of muscle mononuclear cell markers with GM1 in cardiotoxin injected Day 1 gastrocnemius ............................................................................................................. 103

3.22. Double immunostaining of 6 weeks old mdx and Galgt1-/mdx gastrocnemius with GM1 and integrin α7 ......................................................................................................................... 104

xiv
3.23 Double immunostaining of 6 weeks old mdx and Galgt1-/-mdx gastrocnemius with GM1 and CD11b ............................................................ 105

3.24 Double immunostaining of 6 weeks old mdx and Galgt1-/-mdx gastrocnemius with GM1 and Ertr7................................................................. 106
CHAPTER 1. INTRODUCTION.


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1.1. Introduction.

Glycosylation on glycolipids and glycoproteins regulates many maintenance and functional processes in a cell and an organism. Glycosylation can also play central role in pathogenesis of an organism. Almost all cells have an annulus of glycocalyx on their surface with several glycolipids and extracellular proteins being heavily glycosylated with as much as half their molecular weight being represented by glycans. This dense halo of carbohydrates on the cell surface apart from mediating interactions also helps in protection of the cells. The hygroscopic glycans like heparin or heparan sulfates prevents dehydration and can also act as a cushion to protect against mechanical stress. The huge amount of glycocalyx also protects against proteases released by pathogens. The cell surface glycans can, however, be exploited by pathogenic bacteria and viruses.
to gain entry into a host cell. Cholera toxin is one such example where the toxin enters the intestinal muscle by binding to a glycolipid GM1. Glycosylation also mediates immune responses. For example, in the central nervous system (CNS), sialic acid on neuronal surface prevents complement binding and removal by microglia (Linnartz et al., 2012). Glycans also play important role in protein turnover, receptor binding and activation, and in cell adhesion and trafficking. For example, certain immune cells binds to selectins to extravaste from blood vessels to the site of inflammation and this same mechanism is used by cancer cells for mediation of metastatic spread. Glycans mediate cell adhesion and in tumors the glycosylation of surface proteins is reduced as with α-dystroglycan. This reduces the binding of dystroglycan to the extracellular matrix thus promoting metastasis of cancer cells and also causes muscular dystrophy resulting from loss of glycosylation in skeletal muscle. Glycosylation is clearly important in mediation of many biological processes. In this thesis, we have shown the role played by two glycosyltransferases, Galgt1 and Galgt2, and its substrates in muscle biology. In particular, we have looked at the contributions of Galgt2 and Galgt1 in the development of the neuromuscular synapse, where both of these β1-4 GalNAc transferases are localized. We have also shown a role for Galgt1 in skeletal muscle regeneration.

1.2. Glycobiology of Neuromuscular Junction.

While most cells surfaces are heavily glycosylated, the neuromuscular junction has a particularly high density of carbohydrates. Both the pre- and post-synaptic membranes
of the synapse are glycosylated along with the ECM proteins that lie between the two membranes in the synaptic cleft. These glycans mediate important structural as well as biochemical interactions to make a functional neuromuscular synapse. Disturbances of these interactions can lead to pathology. Although the neuromuscular synapse has a wide array of glycans being expressed, some glycans are uniquely synaptic. β-GalNAc is one such carbohydrate that is present only at the neuromuscular junction in a skeletal muscle. This expression has been conserved in many species, ranging from lampreys, the earliest known living vertebrate, to humans. While GalNAc structures can be made by a number of different enzymes and be put onto proteins and lipids via a number of different linkages, two GalNAc transferases that contribute to the expression of synaptic β1,4-linked GalNAc structures are Galgt1 and Galgt2.

1.2.1. Synaptic Glycosyltransferases.

Galgt1 and Galgt2 are two GalNAc transferases expressed at the neuromuscular synapse that are responsible for addition of β-GalNAc to galactose that is linked to a terminal sialic acid.
1.2.1.1. **Galgt1.**

**Figure 1.1 Biosynthesis of GM2 ganglioside.** Galgt1 adds a β1-4 GalNAc moiety (yellow square) to galactose (yellow circle) which is linked to terminal sialic acid (pink diamond) and glucose (blue triangle) which is in turn linked to ceramide.

Galgt1 is a β-GalNAc transferase which is also called GM2/GD2 synthase. Galgt1 adds a β1-4 GalNAc to a galactose moiety that is linked to a terminal sialic acid by a α2-3 linkage and to β1-4 glucose which in turn is linked to ceramide. The addition of β1-4 GalNAc is an important branch point in the synthesis of all complex gangliosides. The glycan structure, Siaα2-3[GalNAcβ1-4]Gal synthesized by Galgt1 is similar to the one synthesized by Galgt2. However, unlike Galgt2, Galgt1 glycosylates only glycolipids and is unable to use glycoprotein glycans as substrate (Rini et al., 2009). In Chapter 2, we
show that Galgt1 is synaptically localized like its products GM2 and GD2. GM2 is primarily present on the pre-synaptic membrane and GD2 on the post-synaptic membrane (Martin et al., 1999). Galgt1 is an important enzyme for normal brain development and function. Galgt1- deficient mice show numerous aging-related CNS and PNS defects, which are usually manifested after the age of 6 months. We have therefore, used mice no more than 3 months old in all our studies. These mice exhibit demyelination of peripheral nerves and decreased axonal diameter probably due to the disruption of myelin-associated glycoprotein (MAG) binding (Pan et al., 2005; Sheikh et al., 1999; Sun et al., 2004), Wallerian degeneration (Sheikh et al., 1999), and motor deficits (Chiavegatto et al., 2000). Galgt1-deficient male mice are sterile; this is probably due to improper spermatogenesis in absence of complex glycosphingolipids. A recent study has shown that these mice also develop Parkinsonism with aging (Wu et al., 2011).
1.2.1.2. Galgt2.

**Figure 1.2. Biosynthesis of CT antigen.** Galgt2 adds a β1-4 GalNAc moiety (yellow square) to galactose (yellow circle) which is linked to terminal sialic acid (pink diamond) and N-acetylgalcosamine (green square), which is ultimately linked to polypeptide chain.

Galgt2 is present at the neuromuscular junction in a skeletal muscle of both humans and rodents (Hoyte et al., 2002; Xia et al., 2002). It is part of the trans-Golgi glycosylation machinery. Galgt2 is an N-acetylgalactosaminyl transferase. It adds a β1-4 GalNAc to a galactose moiety that is linked to a terminal sialic acid by a α2-3 linkage to synthesize a synaptically localized CT antigen, which typically is extended from galactose via N-acetylgalcosamine in a beta 1,4 linkage, as opposed to glucose, which is the next sugar on gangliosides (Martin et al., 1999; Smith and Lowe, 1994). Galgt2 and Galgt1 share the
primary sequence for glycosylation but unlike Galgt1, Galgt2 glycosylates glycoproteins and a non-ganglioside glycolipid. It can glycosylate both N- and O-linked glycoproteins but cannot utilize gangliosides as a substrate (Smith and Lowe, 1994). When overexpressed in skeletal muscle, Galgt2 primarily glycosylates α-dystroglycan (αDG) glycoprotein. The glycosylation of αDG with CT glycan mediates enhances its binding to ECM. Galgt2 can modulate disease pathology in several diseases (See Role of Galgt2 in disease.). Although there is similarity in the glycan structures made by both Galgt1 and Galgt2, they show substrate specificity. But does this formation of similar glycan structures provide functional redundancy? We have addressed this question in Chapter 2 of the thesis.

1.2.2. Synaptic Glycan Structure.

Among the wide array of the glycans present at the neuromuscular junction are the uniquely synaptic products of both Galgt1 and Galgt2. Galgt1 synthesizes synaptic gangliosides GM2/GD2 and Galgt2 synthesizes synaptic CT antigen on glycoproteins.

1.2.2.1. Gangliosides.

Gangliosides are glycosphingolipids with sialic acid moieties attached to them. Glycosphingolipids are oligosaccharide containing sialic acids that are linked to ceramide. Gangliosides have many important functions in maintenance and repair of physiological processes, signal transduction, cell-cell interaction, and cell adhesion. Gangliosides have been implicated in many human diseases. Ganglioside GM1, a
subsequent product of Galgt1, is implicated in the pathogenesis of cholera and influenza. It acts as a receptor for the pathogen. Whereas, these pathogenic diseases are curable in most instances there are more severe neurodegenerative disorders of gangliosides that are lethal with no cure till date. Sandhoff’s and Tay-Sachs disease are autosomal recessive lysosomal storage disorders where β-hexosaminidase enzyme(s) are not synthesized by the cell. This leads to an accumulation of gangliosides, primarily GM2 along with other glycolipids and this is highly pronounced in neurons. This lysosomal accumulation of these metabolites leads to progressive degeneration of the central nervous system, ultimately leading to death. Guillaum-Barre Syndrome is an autoimmune disease that results after a bacterial infection. Antibodies are raised against gangliosides present on the bacterial cell surface which cross-reacts with the gangliosides present on neurons, leading to demyelination and nerve damage. Paralyses, difficulty in breathing, tingling and muscle weakness are some common symptoms of the disease. Apart from playing a role in pathogenesis, complex gangliosides like GM1 have shown to have neuroprotective effects. Intracerebroventricular injection of GM1 in Alzheimer’s patients can lead to improved cognition, improved motor performance, and halt neurodegeneration (Svennerholm et al., 2002). Recently, gangliosides have also been shown to play pivotal role in calcium homeostasis when present on sarcoplasmic reticulum of muscle.
1.2.2.2. Cytotoxic T Cell (CT) Antigen.

The CT glycan, Siaα2-3[GalNAcβ1-4]Galβ1-4GlcNAcβ1-, is synthesized by CT β1-4GalNAc transferase or Galgt2. Galgt2 adds a β1-4 GalNAc to galactose linked to a terminal sialic acid. The sialic acid moiety can be either Neu5Ac or Neu5Gc. Sialic acid, at rodent NMJs, consists of both forms Neu5Ac and Neu5Gc. Humans, by contrast, only synthesize Neu5Ac. The ability to synthesize Neu5Gc was lost after the divergence of humans from chimpanzees due to a deletion in CMP-Neu5Ac hydroxylase gene (Chou et al., 1998). CT antigen was originally identified on activated cytotoxic T cell. Two antibodies, CT1 and CT2, raised against the cytotoxic T cell recognizes this glycan structure (Lefrancois and Bevan, 1985b). The CT1 antibody predominantly stains the presynaptic membrane and recognizes the Neu5Ac form of sialic acid whereas the CT2 antibody predominantly stains the postsynaptic membrane and recognizes the Neu5Gc form (Hoyte et al., 2002; Martin et al., 1999). CT glycosylation of a glycoprotein can mediate its activity and/or function. CT glycosylation of agrin potentiates the AChR clustering activity of neural agrin and imparts the clustering activity to the muscle agrin (Xia and Martin, 2002). It also increases the binding of the extracellular matrix proteins to α dystroglycan (Yoon et al., 2009). Extrasynaptic expression of the CT antigen by transgenic overexpression of Galgt2 protects the sarcolemma from mechanical damage (Yoon et al., 2009) and alleviates dystrophy in several models of muscular dystrophy. It also affects the expression of a number of normally synaptic dystroglycan-binding or -associated proteins.
1.3. Role of Galgt2 in disease.

Transgenic overexpression of Galgt2 in mdx mice (mdx/CT), a mouse model for Duchenne muscular dystrophy, leads to ectopic expression of utrophin, synaptic laminin α-4, laminin α-5, NCAM, and CT-antigen throughout the sarcolemma membrane (Nguyen et al., 2002). α-dystroglycan is highly glycosylated with the CT antigen in such transgenic muscles. The ectopic expression of CT leads to increased expression of other dystroglycan associated proteins. α-, β-, δ, and γ-sarcoglycans, dystrobrevin, utrophin and β-dystroglycan levels in mdx/CT increased (Nguyen et al., 2002), whereas these proteins have reduced levels in mdx mice. Galgt2 overexpression, both embryonically and postnatally, has been shown to ameliorate muscular dystrophy. Galgt2 overexpression not only inhibits muscular dystrophy in DMD mouse model but also in other muscular dystrophy mouse models, including a model for Congenital Muscular Dystrophy 1A (Xu et al., 2007) and for Limb-Girdle Muscular Dystrophy 2D (Xu et al., 2009). This may be in part due to restoration of dystroglycan associated proteins to normal or above normal levels. CT-glycosylated α-dystroglycan also shows increased binding with extracellular matrix protein laminin (Yoon et al., 2009). The increased binding to the extracellular matrix is probably present throughout the sarcolemma membrane with ectopic CT expression. Galgt2 overexpression does not only prevent loss of force during eccentric contraction in mdx mice but also in wild-type (WT) (Martin et al., 2009). This increased binding along with increased expression of dystroglycan
associated proteins probably provides the increased strength and rigidity to the sarcolemmal membrane that is lacking in the dystrophic muscles.

Galglt2 plays a role in not only muscular dystrophies but also in other diseases as well. von Willebrand’s disease, the most common bleeding disorder in humans, can result from altered expression of Galgl2 in endothelial cells. A mutation in Galgt2 gene (Mohlke et al., 1999) leads to a tissue-specific switch from gut epithelial-specific expression to a vascular endothelial specific expression with loss of gut epithelial expression. This altered expression causes aberrant glycosylation of vWF (von Willebrand factor) which leads to its premature clearance from the blood plasma. vWF is essential for clot formation and acts like a bridging factor between platelets and the wall of the blood vessel. Gastrointestinal cancers in humans and mice have reduced expression of CT antigen by Galgt2. An increase in Galgt2 leading to an increase in CT antigen expression reduces the interaction of the cells with selectins and thus reduces the metastasis of the cancer cells (Kawamura et al., 2005). Thus, Galgt2 is a therapeutic gene target in diseases besides muscular dystrophy. In this thesis, we have tried to elucidate Galgt2’s normal function at the neuromuscular junction.

1.4. Neuromuscular Junction and its Development.

Our understanding of the formation of the neuromuscular synapse began, in large part, with the approach of assessing motor axon or muscle regeneration after injury in the adult animal. The neuromuscular synapse has been a favorite of developmental
neurobiologists for three reasons: 1. Its large size, about 1000 times that of a typical interneuronal synapse, 2. Its simplicity of patterning, almost all mammalian synapses are ultimately innervated by only one nerve terminal, and 3. Its experimental accessibility, which allows imaging and cell manipulation in the living animal. Of course, being an essential synapse that controls voluntary movement and breathing, the NMJ is also very important in its own right. Neuromuscular pathology and dysfunction are involved in a number of human diseases, for example the congenital and autoimmune myasthenias (Engel et al., 2010; Farrugia and Vincent, 2010). In mammals, including rodents and humans, the NMJ utilizes cholinergic neurotransmission to control muscle movement. Acetylcholine made in motor neurons is packaged into secretory vesicles that reside within the nerve terminal. When the motor nerve, whose cell body resides within the ventral column of the spinal cord or the hindbrain, is excited, an action potential is generated, leading to quantal release of acetylcholine at the NMJ. Nicotinic acetylcholine receptors (AChRs) concentrated in the postsynaptic membrane are activated upon binding acetylcholine to depolarize the muscle membrane, with elevated intracellular calcium levels ultimately causing muscle contraction.

To bring such a robust synaptic structure about, a large number of developmental events must take place. As the NMJ matures, its shape changes on several levels (Sanes and Lichtman, 2001): 1. The synaptic membrane transforms from a contiguous immature oval plaque to a disjointed pattern of pretzel-like regions. 2. The topography of the pre- and postsynaptic membrane is altered, such that infoldings of
the postsynaptic membrane, called secondary folds, align with concentrations of secretory vesicles docked in specialized presynaptic membrane regions called active zones. 3. The extracellular matrix surrounding each skeletal myofiber and within the synaptic cleft forms and matures, both in terms of its density and its molecular constituents, 4. Motor axons that have multiply innervated single myofibers in the embryo are eliminated postnatally such that most myofibers are monoinnervated in the adult, and 5. A synaptic non-myelinating Schwann cell covers the nerve terminal in areas outside the synaptic cleft, capping the region of contact between the nerve terminal and the muscle membrane. These changes coincide with developmental changes that are further removed from the NMJ (sarcomeric patterning and determination of muscle fiber type, muscle growth, adjoining of myofibers to tendons at myotendinous junctions, myelination of motor axons by Schwann cells and development of motor neuron subtypes and pools).
Figure 1.3. Developmental expression of synaptic extracellular matrix (ECM) proteins at the rodent neuromuscular junction. By embryonic (E) day 15, skeletal myofibers have formed and are innervated the nerve terminals of motor neurons at the neuromuscular junction. The nerve terminal is capped by a non-myelinating Schwann cell. Skeletal myofibers express a number of ECM proteins, including laminins, and collagens, while motor nerves express ECM proteins including agrin (z8) and neuregulin. Some ECM proteins are localized within the synaptic (SYN) basal lamina, which exists only at the neuromuscular junction, while other ECM proteins are present within the extrasynaptic (ESYN) basal lamina that surrounds the remainder of the skeletal myofiber membrane. ESYN and SYN composition shifts during neuromuscular development as synaptic ultrastructure develops, such that unique laminin and collagen chains become expressed exclusively in the synaptic basal lamina in the postnatal (P) animal.
1.4.1. Acetylcholine Clustering.

The most striking aspect of NMJ development is the intense concentration of postsynaptic acetylcholine receptors, or AChRs, in the muscle membrane underlying the nerve terminal. During embryogenesis, as myoblasts fuse to form myotubes, synaptic genes, including the subunits of the AChR, are activated to produce high concentrations
of AChRs (about 1000/µm²). Through a combination of mechanisms (lateral protein migration to synapses, increased mRNA biosynthesis by subsynaptic muscle nuclei, increased protein stability in synaptic regions, increased protein degradation and reduced mRNA expression in non-synaptic regions), AChRs are reorganized and concentrated such that AChR density is 10,000-20,000/µm² at the adult NMJ while in extrasynaptic regions AChR density is less than 10/µm² (Fambrough, 1979; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001). In addition, AChR subunit composition is altered, with α₂βδε pentameric AChR channels present in the embryo and α₂βδγ channels present in the adult (Sunesen and Changeux, 2003). Changed AChR subunit composition alters AChR calcium permeability and channel open time. This allows the nerve to excite the skeletal myofiber to membrane potentials well beyond those required to elicit contraction, providing a safety factor for a synapse that must work with high efficiency for the organism to survive.
1.4.1.1. Agrin.

Figure 1.4. Synaptic extracellular matrix (ECM) protein agrin and its receptors at the neuromuscular junction. Agrin contains splice sites in the N-terminus to allow for secreted and transmembrane forms and A/y and B/z splice sites in the C-terminus that allow heparin binding and AChR clustering, respectively. Potential ECM-receptor and ECM-ECM interactions are indicated in blue. Legend: EGF, Epidermal growth factor-like repeat; FS, Follistatin-like domain; LE, laminin EGF-like domain; LG, laminin-like globular domain; NtA, N-terminal agrin-laminin binding domain; S/T, serine/threonine-rich mucin-like domain; SP, signal peptide; SEA, sea urchin sperm protein, enterokinase, and agrin domain; TM, transmembrane domain; NCAM, neural cell adhesion molecule; FGF, fibroblast growth factor; Lrp4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase.

Agrin is a 400 kDa heparan sulfate proteoglycan which was originally purified from the marine ray, *Torpedo californica* (Godfrey et al., 1984; Nitkin et al., 1987; Wallace et al., 1985). Agrin is heavily glycosylated with one-half of its weight being that of carbohydrates. It contains GAG chains, N-linked glycosylation and O-linked
carbohydrates that are present on mucin-like regions (Rupp et al., 1991; Tsen et al., 1995; Tsim et al., 1992). Agrin is 95nm long with globular N- and C-terminals and a central rod like domain (Denzer et al., 1998). This multidomain protein has a unique splice site at the N-terminus and can either be anchored to the plasma membrane of neuron via the transmembrane domain or can be secreted into the synaptic cleft by the nerve terminal (Burgess et al., 1999; Burgess et al., 2000; Denzer et al., 1998; Magill-Solc and McMahan, 1990a; Magill-Solc and McMahan, 1990b). The NtA domain at the N-terminus binds to laminin which helps agrin to adhere to the extracellular matrix (ECM), this adhesion to ECM is important for postsynaptic differentiation and maintenance of the high acetylcholine receptor (AChR) concentration at the neuromuscular junction (NMJ) (Denzer et al., 1998; Rupp et al., 1991; Tsim et al., 1992). The central rod-like domain contains nine follistatin-like repeats (FS) which are homologous to Kazal-type of protease inhibitors (Denzer et al., 1998; Rupp et al., 1992; Rupp et al., 1991). It also contains serine/threonine (S/T) rich regions that are similar to mucin domains which contains O-linked glycosylations (Rupp et al., 1991). The FS and S/T regions are near amino acids that can be modified to be glycosylated with heparan sulfate glycosaminoglycans (GAG) chains that bind to fibroblast growth factor 2, NCAM, thrombospondin (Cole and Halfter, 1996; Cotman et al., 1999). The C-terminal of agrin contains four EGF and three LG domains (Ruegg et al., 1992; Rupp et al., 1991; Tsim et al., 1992). There are two splice sites present in agrin; the y splice site is present in LG2 and the z splice site in LG3 domain. The y splice site encodes a four amino acid insert
and the z splice site, which can allow for one or two introduced exons, can encode an 8, 11, or 19 amino acid insert (Ferns et al., 1992; Ferns et al., 1993; Hoch et al., 1993). The y splice mediates agrin heparin interaction and the z splice site requires an insertion of one or both exons for AChR clustering activity in vitro. LG3 apart from being essential for AChR clustering also binds to sodium-potassium ATPase (Campanelli et al., 1996; Gesemann et al., 1996; Gesemann et al., 1995). LG2 binds to α-dystroglycan and can also change the potency of agrin to induce AChR clustering (Bowe et al., 1994; Campanelli et al., 1996; Gesemann et al., 1998; Gesemann et al., 1996). One of the EGF domains or agrin is glycosylated with Pofut1. O-fucosylation of this domain by Pofut1 changes the biological activity of muscle agrin (Kim et al., 2008a). Agrin also binds to integrins (Burgess et al., 2002). Agrin, thus, by virtue of its diverse binding partners is a very important mediator of neuromuscular synapse development and maintenance.

Agrin was first discovered by McMahan and colleagues for its AChR clustering activity (Godfrey et al., 1984; McMahan, 1990; Nitkin et al., 1987; Wallace et al., 1985). They observed that agrin when added to myotubes induced AChR clustering without changing the total number of AChRs on the myotube surface or the rate of AChR degradation. This observation lead to the hypothesis that agrin acts as an inducer of AChR aggregation embryonically and helps in maintaining the post-synaptic density of AChRs at an adult NMJ (McMahan, 1990). However, later studies pointed to a role of agrin as a synaptic organizer/ stabilizer rather than AChR clustering inducer. Agrin deficient mice have disorganized NMJs with numerous aberrations in both pre- and
post-synaptic membranes. The AChR aggregates are reduced in size, number and
density in the postsynaptic membrane. There is decreased apposition between the pre-
and post-synaptic membranes. The motor nerve branching increases and the nerve
branches are no longer restricted to the central end plate region. There are fewer
vesicles in the nerve terminal and the nerve terminal is loosely associated with the
Schwann cells (Burgess et al., 1999; Gautam et al., 1996). These observations showed
that agrin does act as a critical organizer of both pre- and post-synaptic membrane.
Studies on mice lacking the motor nerve further proved that agrin acts to stabilize
already prepatterned AChR receptor clusters on muscle fibers. Deletion of HB9 (Lin et
al., 2001; Yang et al., 2001), motor neuron specific transcription factor, and DNA
topoisormerase Iiβ (Yang et al., 2000) prevented the phrenic nerve, which normally
innervates diaphragm, from innervating the muscle fibers. These mice in which the
motor nerve failed to contact the muscle fibers still had clusters of AChRs that were
present in a broader central end plate region. In a normal rodent NMJ, after the nerve
innervates the muscle fiber, the AChRs are concentrated in a much narrower end plate
region and the extrasynaptic clusters of AChRs dissipate at birth. This, however, was
prevented in ChAT deficient mice (Misgeld et al., 2002). ChAT, choline acetyltransferase,
is an enzyme required for biosynthesis of acetylcholine (ACh). ACh is the
neurotransmitter released by motor nerve to depolarize the muscle membrane causing
it to contract. The deficiency of ChAT in agrin mutant mice rescued the postsynaptic
aberrations in the agrin null mice. In the double knockout mice, the density of AChR
clusters at the synapse is maintained, the synapse is differentiated and innervation is more normal as compared to agrin and ChAT single knockout. These studies show that ACh destabilizes the postsynaptic specializations and that agrin counteracts this by stabilizing the AChRs at the synapse (Misgeld et al., 2005). This dispersal of AChRs from the extrasynaptic membrane in response to the muscle activity is probably mediated by several mechanisms acting in conjunction. Myogenin is required for AChR upregulation but during muscle activity (Durr et al., 1994; Gundersen et al., 1995; Gundersen et al., 1993), in response to ACh, calcium/calmodulin-dependent protein kinase II phosphorylates myogenin which affects its activity as a transcription factor leading to inhibition of AChR gene expression (Tang et al., 2004). Cyclin-dependent kinase 5 (cdk5) is activated by ACh as well and leads to dispersal of AChRs in absence of agrin. In agrin deficient mice, blockage of cdk5 leads to retention of AChR clusters (Fu et al., 2005; Lin et al., 2005). Calpain, a calcium-dependent protease, is also required for dispersal of AChRs upon activation with ACh. The activity of calpain can be inhibited by its interaction with rapsyn in presence of agrin (Chen et al., 2007). Thus, at NMJ neural agrin stabilizes the receptor clusters whereas at the extrasynaptic membrane where agrin is absent, the receptor clusters disperse with muscle activity.
Figure 1.5. Synaptic ECM signaling in neuromuscular development. Signals from neuron-specific splice forms of agrin (e.g., z8) in the synaptic basal lamina (BL) work via the MuSK-LRP4 complex in the membrane. It induces downstream tyrosine phosphorylation signals that drive synaptogenesis, which is agrin-independent, and to organize synaptic topography and induce synaptic stabilization, which are agrin-dependent. Down-stream signals are mediated or modulated by a variety of cytoplasmic effectors, including structural and adaptor/signaling proteins (Dok7, Dvl, Pak1, Tid1, APC, Rapsyn), proteases (Calpain), tyrosine kinases (Abl, Src, Fyn), tyrosine phosphatases (SHP2), serine/threonine kinases (Cdk5, CaMKII and CK2), small G proteins (Rho, Rac), and a geranylgeranyltransferase (GGT). Calcium entry stimulated by acetylcholine works, in part via Calpain, Cdk5, and CamKII to destabilize synaptic membranes. Laminins, particularly Laminin 421 and 521, in the synaptic BL signal via dystroglycan and possibly integrins in muscle to induce changes in synaptic ultrastructure and to maintain synaptic stability. Ultrastructural changes at the synapse are mediated, in part, by cytoplasmic dystroglycan-binding proteins including utrophin, syntrophins, and dystrobrevins. These proteins help to link the ECM outside the muscle membrane to filamentous (F-) actin in the cytoskeleton. Laminin 421 and 521 also bind to the voltage-gated calcium channels in the presynaptic membrane to organize active zones. Collagens IV in the synaptic BL and collagen XIII in the postsynaptic membrane may signal via integrins to induce synaptic maturation and stability. Integrins may bind and signal through a variety of intracellular proteins [e.g., Focal adhesion kinase (FAK), vinculin, paxillin, talin].
1.4.1.2. MuSK.

MuSK is a tyrosine kinase (Jennings et al., 1993) which acts as a receptor for agrin (DeChiara et al., 1996; Glass et al., 1996). Agrin does not directly bind to MuSK (Glass et al., 1996) but rather binds to Lrp4 (Kim et al., 2008b; Zhang et al., 2008) and activates MuSK via agrin-Lrp4-Musk signaling. Lrp4 is a densely glycosylated (May et al., 2007) low-density transmembrane lipoprotein receptor that is present at NMJ. The mice lacking Lrp4 die at birth and have defects in both pre- and post-synaptic membrane specializations. The mice lack concentration of AChR at the NMJ along with the selective sub-synaptic expression (Weatherbee et al., 2006). These findings are similar to MuSK knockout mice, where no NMJs are formed (DeChiara et al., 1996; Glass et al., 1996).
Further, expression of Lrp4 can restore MuSK autophosphorylation after agrin application (Kim et al., 2008b; Zhang et al., 2008). Overexpression of MuSK causes ectopic AChR aggregation in absence of agrin (Kim and Burden, 2008) and agrin can no longer cause AChR clustering in MuSK deficient myotubes (Glass et al., 1996; Zhou et al., 1999). These findings lead to the hypothesis that MuSK pre-patterns the post-synaptic membrane before the nerve arrives and agrin activates MuSK and its downstream signaling cascade through binding to Lrp4. Binding of agrin to Lrp4 causes Musk autophosphorylation at 553 tyrosine residue near the transmembrane region (Watty et al., 2000), thus recruiting Dok7 dimer which cross-links Musk dimer (Herbst et al., 2002; Herbst and Burden, 2000; Till et al., 2002). MuSK then phosphorylates itself at 750, 754, and 755. This probably lifts the auto-inhibition from the cytoplasmic region which then facilitates substrate binding (Till et al., 2002; Watty et al., 2000). Casein kinase 2 (ck2) phosphorylates MuSK which leads to AChR clustering (Cheusova et al., 2006). Src and Fyn’s phosphorylation of AChR-β subunit depends on MuSK as well (Mittaud et al., 2004; Sadasivam et al., 2005; Smith et al., 2001). Thus, MuSK plays a central role in AChR clustering and pre-patterning the post-synaptic membrane. These pre-patterned aggregates that are centrally located on the muscle fibers are then innervated by the nerve and stabilized by agrin.

1.4.1.3. Neuregulin.

The high density concentration of AChRs at the neuromuscular synapse occurs due to AChR clustering, stabilization, and selective expression by the sub-synaptic nuclei.
Neuregulin, originally called as AChR-inducing activity (ARIA) was thought to be the factor driving the selective sub-synaptic expression of AChRS (Chu et al., 1995; Falls et al., 1993). Neuregulin is synthesized and secreted into the synaptic ECM by the motor neuron (Sandrock et al., 1995; Usdin and Fischbach, 1986). It signals through binding dimeric epidermal growth factor receptor tyrosine kinases, erbB2-erbB3 or erbB2-erbB4 (Gassmann and Lemke, 1997). Neuregulin-erbB signaling is critical for nerve-dependent Schwann cell survival and functioning, which in turn is essential for axonal growth and differentiation (Lemke, 2006; Trachtenberg and Thompson, 1996). To overcome this limitation in the experiments where aberrations in NMJs of were observed in heterologous neuregulin-1 mutants (Liu et al., 2005) and erb2 or erb3 mutants in which cardiac defects were rescued (Woldeyesus et al., 1999), conditional knockouts of neuregulin (both motor neuronal and skeletal muscle specific) and skeletal muscle specific erbB2 and/or erbB4 were made (Escher et al., 2005; Jaworski and Burden, 2006). In these skeletal muscle-specific knockout mice, the Schwann cell and cardiac defects were spared and only the skeletal muscle signaling will be affected (Sanes and Lichtman, 2001). These mice had normal muscle strength, relatively normal NMJ development with slightly reduced AChR density. At best, neuregulin plays a modulatory role instead of being the primary inducer of AChR transcription as was indicated in the early studies.
1.4.2. Synaptic Carbohydrates and the DAG complex.

The neuromuscular synapse contains a large collection of very densely packed glycoproteins and glycolipids that contribute to this highly specialized structure. There are numerous glycoproteins that are present at the synapse and the glycosylation is essential for their interaction with binding partners. Agrin is one such proteoglycan which is heavily glycosylated with about half of its weight coming from carbohydrates. So it is not surprising that these carbohydrates are important implications in agrin function. Clustering of AChRs is essential for a functional neuromuscular junction. This important process requires many signaling and scaffolding molecules to work together. Agrin is the main organizer that is essential to maintain and stabilize the high concentration of AChRs at the NMJ. Since agrin is a heavily glycosylated glycoprotein like some of its binding partners, it is plausible that these interactions are mediated by carbohydrates. Previous studies have shown that heparin and heparan sulfate can block agrin mediated AChR clustering. Heparin can also inhibit agrin binding to α-dystroglycan (Campanelli et al., 1996; Gesemann et al., 1996; Wallace, 1990). Further, cells deficient in biosynthesis of glycosaminoglycans have decreased potency of agrin to induce AChR clusters and decreased agrin binding to α-dystroglycan (Gordon et al., 1993; Sugiyama et al., 1994). Agrin also gets glycosylated by CT antigen which increases its AChR clustering activity and binding to myotubes. N-terminal to the three LG domains in agrin, is a serine/threonine rich mucin domain which is essential for CT glycosylation of the LG2 domain, which also binds α-dystroglycan. The mucin domain inhibits AChR
clustering by agrin but CT glycosylation of the mucin domain overturns the inhibition (Xia and Martin, 2002). CT antigen is present at the NMJ; it has a terminal GalNAc that is linked to galactose in a β 1-4 linkage. Localization of the terminal GalNAcs to the neuromuscular synapse is phylogenetically conserved across species. Martin et al showed that a plant lectin VVA-B4 which binds to GalNAc stains the spontaneously generated AChR clusters on myotubes showing that GalNAc is present in the AChR aggregates. It was also shown that VVA-B4 addition to myotubes induces AChR clustering and that this clustering is blocked by addition of GalNAc. VVA-B4 also potentiated the effect of agrin in AChR clustering. Treatment of myotubes with enzymes that removed terminal β-GalNAc from the cell surface inhibited agrin-induced AChR clustering (Martin and Sanes, 1995). These results indicated that agrin acts in a GalNAc dependent manner and in absence of terminal β-GalNAc was unable to induce AChR clustering. Other studies showed that unmasking of certain glycan structures can also induce AChR clustering independent of agrin (Martin and Sanes, 1995; Parkhomovskiy and Martin, 2000). These studies laid a strong foundation to argue that carbohydrates play important role in formation, functioning, and maintenance of the synapse.

1.5. **Skeletal Muscle Regeneration.**

Skeletal muscle is remarkably complex and highly specialized organ system. Skeletal muscle primarily provides locomotion in animals which from evolutionary stand point is critical for survival. It also gives strength to fight and engage in normal day-to-day
activity. Since effective working of skeletal muscle is central to survival, it’s not surprising that it has an extraordinary capacity to regenerate itself. Skeletal muscle regenerates in response to stretch stimulus from exercising or in response to damage caused by insult or injury. In 1917, Lewis and Lewis observed that skeletal muscle grew in size and number of myonuclei without any apparent nuclear division. However, it took several decades after the initial observation to finally understand in 1961 that muscle grew by fusion of large numbers of mononuclear cells, now known as satellite cells (Cooper and Konigsberg, 1961; Stockdale and Holtzer, 1961). These cells, as shown by electron microscopy, reside next to the myofiber and have a distinct heterochromatin pattern within their nuclei (Mauro, 1961). Satellite cell shares the basal lamina with myofiber and is the primary myogenic stem cell population present in the muscle. They have the ability to undergo asymmetrical division to give rise to daughter cells that can differentiate to form myoblasts or can self-renew to form more satellite cells (Kuang et al., 2007). The process of regeneration in mammalian skeletal muscle is very extensively studied. There are various methods of creating a model for muscle regeneration, but snake venom toxins, such as cardiotoxin, are most commonly used. Cardiotoxin is extracted from Naja mossambica mossambica. Cardiotoxin binds to the calcium channel in the skeletal myofiber membrane and opens it which, ultimately leading to degeneration of the muscle fiber and stimulating the regenerative process to occur (Couteaux et al., 1988; Duchen et al., 1974). Muscle regeneration resulting from acute injury is essentially similar to mechanisms that occur during normal muscle
development, with a few differences. In developmental myogenesis, as myofibers mature the centrally placed myonuclei move to the periphery. For reasons that have never been fully explained, this fails to occur during post-natal muscle regeneration in dystrophic animals (Charge and Rudnicki, 2004). Centrally placed nuclei therefore, can be a useful tool to measure muscle regeneration in such models. The necrotic changes take place shortly after administration of cardiotoxin. On day 1 there is a massive infiltration of immune cells in the muscle and an increase in the number of macrophages. They help in clear cellular debris. By day 2, spindle shaped activated satellite cells are observed along with the necrotic debris and immune cells. By day 3, initiation of myotube formation can be observed, and by day 7, most of the muscle has regenerated and the fiber is still growing in girth. By 1 month the regeneration process is essentially complete with myofiber maturation and increase in size (Couteaux et al., 1988; d’Albis et al., 1988; Whalen et al., 1990). Satellite cells are the primary myogenic stem cells in the muscle but other types of cells can contribute towards myofiber regeneration and maturation. Following are the highlights of the major cell types involved in muscle regeneration. A detailed overview of these cell types is beyond the scope of this thesis.

1.5.1. Satellite Cells.

Satellite cells are myogenic stem cell population in skeletal muscle responsible for proliferating, differentiating, and fusing to form new myofibers. These cells also have a
tremendous capacity to regenerate themselves. On average, an adult human satellite cell can produce fusion competent progeny through 25-30 cycles of doubling in vitro (Ham et al., 1990; Snow, 1977). 30 doublings can yield more than $10^9$ cells. This makes the muscle a resilient regenerating organ. Satellite cells received their name due to their positioning on the myofiber (Mauro, 1961). They are spindle shaped and present next to the myofiber, under the basal lamina. Thus, the myofiber and the associated satellite cells are enwrapped within the same basal lamina. There are about 20 times more satellite cells near the NMJ as compared to elsewhere on the fiber (Kelly, 1978a). Similarly, slow-twitch muscles contain about 3-4 times more satellite cells as compared to the fast-twitch muscles (Gibson and Schultz, 1982; Kelly, 1978b). The reason for this difference between the satellite cell populations according to the muscle type isn’t clear. Satellite cells are thought to arise from the embryonic skeletal muscle progenitor cells (Armand et al., 1983; Gros et al., 2005). These cells originate from the paraxial mesoderm. Paraxial mesoderm undergoes segmentation to give rise to somites, which in turn gives rise to satellite cells (Ben-Yair and Kalcheim, 2005). The progenitor cells that give rise to satellite cells express Pax3/Pax7 (Pax3/7) in the dermomyotome (Kassar-Duchossoy et al., 2005; Relaix et al., 2006; Relaix et al., 2005). These progenitor cells lack other myogenic-regulatory factors (MRFs) like Myf5, MyoD, myogenin etc (Kuang and Rudnicki, 2008). These uncommitted Pax3/7$^+$MRF$^-$ cells proliferate throughout embryonic myogenesis and can become committed to myogenic fate by expressing Myf5 or MyoD. These cells align against the nascent myotube by E15.5 and
take sublaminar position to become satellite cells (Relaix et al., 2005). Once in sublaminar position, these cells downregulate Pax3 and upregulate Myf5 (Kassar-Duchossoy et al., 2005). When activated, these Pax7+/Myf5+ cells divide asymmetrically for self-renewal and to give rise to committed myoblasts (Kuang et al., 2007). However, a small portion of satellite cells are Pax7+/Myf5− which can divide asymmetrically like satellite cells to give rise to self-renewal cell or a committed myoblast or can give rise to two identical self-renewal cells (Kuang et al., 2007). Therefore, these cells have more primitive stem cell character than Pax7+/Myf5+ satellite cell. Pax7+/Myf5+ satellite cells are in quiescence and will remain to be so until activated by exercise, mechanical or chemical trauma. Satellite cells get activated by producing sphingosine-1-phosphate (Loh et al., 2012; Nagata et al., 2006). Upon activation Pax7+/Myf5+ cells upregulate MyoD. These cells can then either differentiates to become a committed myoblast by downregulating Pax7 and upregulating myogenin or downregulating MyoD to return to quiescence. The differentiated myoblasts expressing myogenin also express myosin heavy chain to form myocytes which fuse together to form myotubes (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004).

Satellite cells move far distances across the myofiber and also across the basal lamina to repair or regenerate myofibers. The basal lamina in adult is thicker than in a young animal (Kovanen et al., 1988) and this may pose a barrier for satellite cell migration across the basal lamina. In 2-3 weeks old rats, the satellite cell progeny can migrate across the basal lamina and fuse with neighboring myofibers (Kennedy et al.,
The efficiency of migration of satellite cells increases after damage to the basal lamina (Bischoff, 1974; Bischoff, 1975; Schultz et al., 1986). This translaminar movement can also be observed after muscle damage caused by snake venom (Klein-Ogus and Harris, 1983; Maltin et al., 1983). The motility of the cells decreases with differentiation. Myoblasts are more motile than myocytes (Griffin et al., 2010; Powell, 1973). The migration and subsequent fusion of myogenic cells is a result of many positive and negative factors acting in concert. Myoblasts also respond to chemotactic signals (Corti et al., 2001). Successful muscle regeneration requires the myoblasts to contact, adherence, and fusion. Myoblasts make contact with each other through lamellopodia or filopodia (Mukai et al., 2009; Stadler et al., 2010; Yoon et al., 2007). The adhesion and fusion of the myoblasts is mediated by several adhesion molecules like M-cadherin (Cifuentes-Diaz et al., 1995), β-catenin (Mukai et al., 2009), integrins (Brzoska et al., 2006; Lafuste et al., 2005; Schwander et al., 2003) and by cholesterol rich lipid rafts (Mukai et al., 2009). The adhesion molecules are concentrated near lipid rafts at the point of contact and are dispersed with cholesterol depletion. Cholera toxin subunit B binds to GM1 ganglioside and enhances myoblast fusion in vitro (Mukai et al., 2009). A negative regulator of myoblast fusion is ADAM 12 that inhibits myoblasts from binding myocytes (Lafuste et al., 2005). Satellite cells also possess the ability to give rise to osteogenic as well as adipogenic cells after treatment with appropriate factors (Asakura et al., 2001).
1.5.2. Other Cell Types That Contribute to Regeneration.

Many studies have pointed that there are other cell types in skeletal muscle apart from satellite cells that have myogenic potential. The contribution of these cells in a normal muscle during regeneration and repair is controversial; their ability to repair or regenerate muscle can, however, be exploited in a diseased state where regeneration fails with the failure of satellite cell’s regenerative ability. A number of cell types in the muscle have shown the ability to differentiate into muscle tissue; side populations cells (SP), muscle derived stem cells (MDSC), pericyte, and bone marrow stem cells. SP cells are a part of muscle-derived stem cells based on Hoechst dye exclusion. SP cells display the property of giving rise to myogenic cells \textit{in vivo} and undergo myogenic specification after being co-cultures with myoblasts \textit{in vitro} (Asakura et al., 2002). Pericytes are cells that are associated with the blood vessels and are present outside the basement membrane. They are related to embryonic mesoangioblasts. These cells have been shown to differentiate into myotubes very efficiently both \textit{in vitro} and \textit{in vivo}. The cells can cross the endothelium of the muscle and can be delivered intra-arterially to give rise to myofibers in muscular dystrophy model (Dellavalle et al., 2007). Bone marrow stem cells can also contribute to muscle regeneration. In bone marrow transplant studies in mdx mice, the donor cells were incorporated into the muscle fibers around 8-12 weeks after transplantation (Gussoni et al., 1999).
CHAPTER 2. Distinct contributions of Galgt1 and Galgt2 to synaptic carbohydrate expression and function at the mouse neuromuscular junction.

2.1. Introduction.

Synapses contain large collections of proteins and lipids linked together to form specialized complexes within the pre- and postsynaptic cellular membranes, and cell surface carbohydrates can play important roles in defining the localization and function of such synaptic molecules (Dani and Broadie, 2012; Martin, 2002; Martin, 2003a; Martin, 2003b; Martin and Freeze, 2003; Rushton et al., 2012). At the vertebrate neuromuscular junction (NMJ), the synaptic connection made between the nerve terminal of motor neurons and skeletal myofibers, there are several carbohydrates known that may participate in aspects of synaptic development and function (Dani and Broadie, 2012; Hoyte et al., 2002; Jenniskens et al., 2000; Martin et al., 1999; Sanes and Cheney, 1982; Scott et al., 1988; Singhal and Martin, 2011). One such structure is the Cytotoxic T cell (CT) carbohydrate, Neu5Ac/Gcα2,3[GalNAcβ1,4]Galβ1,4GlcNAcβ-R (Conzelmann and Lefrancois, 1988), also called the Sda or Cad blood group antigen in
humans (Conzelmann and Lefrancois, 1988; Montiel et al., 2003). The localized muscle expression of the CT carbohydrate at the NMJ results from the presence of a β1,4-linked N-acetylgalactosamine (βGalNAc). Terminal β-linked GalNAc structures are confined to the NMJ in mammals including mice, rats, hamsters, guinea pigs, dogs, cats, monkeys and humans, as well as in goldfish, frogs, chickens, axolotls, snakes and lampreys (Martin, 2003b; Sanes and Cheney, 1982; Scott et al., 1988). The form of sialic acid on the CT carbohydrate helps to contribute to pre- vs. post-synaptic specificity; CT2, a monoclonal anti-CT antibody that recognizes Neu5Gcα2,3[GalNAcβ1,4]Galβ1,4GlcNAc-R, stains primarily the postsynaptic membrane, while CT1, which recognizes Neu5Acα2,3[GalNAcβ1,4]Galβ1,4GlcNAc-R, primarily stains the presynaptic membrane (Hoyte et al., 2002; Martin et al., 1999).

The synaptic portion of the CT carbohydrate is synthesized by Galgt2, a β1,4GalNAc transferase whose expression is also highly confined to synaptic regions in skeletal myofibers (Smith and Lowe, 1994; Xia et al., 2002). Galgt2 is known to glycosylate only a small set of glycoproteins, including α dystroglycan and non-GM2/GD2 glycolipid in skeletal muscle (Kawamura et al., 2005; Lefrancois and Bevan, 1985a; Nguyen et al., 2002; Xia et al., 2002; Xia and Martin, 2002; Xu et al., 2007; Yoon et al., 2009). By contrast, Galgt1, also called the GM2/GD2 synthase, is a β1,4GalNAc transferase specific for ganglioside glycolipid substrates and is required for the synthesis of all complex gangliosides (Takamiya et al., 1996). Because the GM2 (Neu5Ac/Gcα2,3[GalNAcβ1,4]Galβ1,4Glc-Ceramide) and GD2 gangliosides, like the CT
carbohydrates that occur on proteins, are localized to the NMJ (Martin et al., 1999), there is the potential for redundancy between these groups with regard to neuromuscular development. In addition, both Galgt1 and Galgt2 can contribute to expression of the CT carbohydrates as defined by antibody staining (Martin et al., 1999). Here, we use genetic methods in the mouse to dissect the relative contributions of Galgt1 and Galgt2 to the synthesis of synaptic CT carbohydrates at the NMJ and determine the relative impact of their overexpression, and loss of expression, on neuromuscular development.

2.2. Materials and Methods

2.2.1. Materials

Monoclonal antibody to laminin α2 was obtained from Alexis Biochemicals (San Diego, CA). Antibody to α dystroglycan (IIH6) was obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies to utrophin (DRP2), dystrophin (DYS1), β dystroglycan (43DAG) and α sarcoglycan (α-SARC) were obtained from Nova Castra (Newcastle-Upon-Tyne, UK). Antiserum to NCAM, laminin α4 and laminin α5 were gifts from Joshua Sanes (Harvard University), Bruce Patton (Oregon Health Sciences University) and Jeffery Miner (Washington University), respectively. Antiserum to agrin was a gift from Michael Ferns (UC Davis). Affinity purified antiserum to acetylcholinesterase was a gift from Palmer Taylor (UC San Diego). Additional antibody to laminin α2 was from Sigma-Aldrich (L0663), as were antibodies to calnexin (C4731)
and Lamp1 (L1418). Antibody to clathrin heavy chain was from Cell Signaling (2410) and antibody to TGN38 was from Serotec (AHP499G). Rhodamine-α-bungarotoxin was purchased from Molecular Probes (Eugene, OR). Secondary antibodies conjugated to fluorophores were purchased from Jackson ImmunoResearch (West Grove, PA).

Antibodies to CT1, CT2 and Galgt2 were produced in our lab as previously described (Martin et al., 1999; Xia et al., 2002). Rabbit polyclonal antibody to Galgt1 peptide CQVRAVDLTKAFDAEE, was made by immunizing rabbits with KLH-conjugated peptide, after which antibody was purified over peptide conjugated resin as previously described (Xia et al., 2002).

2.2.2. Mice

Mice lacking Galgt2 (Galgt2-/-) were made by John Lowe (Genentech, South San Francisco CA) and mice lacking Galgt1 (Galgt1-/-) were obtained from the Consortium for Functional Glycomics. Double mutant mice (Galgt1-/-Galgt2-/-) were made by interbreeding the two lines on a pure C57Bl/6 genetic background.

Mice lacking dystroglycan (Dag1) in caudal but not rostral muscles (Jarad and Miner, 2009) were made by breeding P3Pro-Cre transgenic mice (originally made by Epstein and colleagues, U. Pennsylvania (Li et al., 2000)) to Dag1^loxP/loxP mice (originally made by Campbell and colleagues, U. Iowa (Cohn et al., 2002; Moore et al., 2002)).
2.2.3. Histology

Whole mounts of diaphragm were done as previously described by us (Xia et al., 2002).

For immunostaining of thin sections, mouse muscles were snap frozen in liquid nitrogen-cooled isopentane. For analysis of brains and spinal cords, mice were exsanguinized under anesthetic and fixed in 4% paraformaldehyde. Tissues were dissected, dehydrated in sucrose solutions, placed in OCT freezing medium and frozen in dry ice/isopentane slurry. All tissues were sectioned (at 10µm for brain and spinal cord and at 8 µm for all other tissues) on a cryostat and mounted on gelatin-coated glass slides. Tissue sections were blocked in phosphobuffered saline (PBS) with 3mg/ml bovine serum albumin (BSA) for 1 hour at room temperature and incubated with primary antibody for 1-2 hours at room temperature (or overnight at 4°C). Tissue sections were washed (4 times for 10 minutes each) in PBS and incubated in PBS/BSA with 10µg/ml of the appropriate FITC-coupled secondary antibody for one hour.

Skeletal muscle sections were also incubated with 50nM rhodamine-α-bungarotoxin to identify acetylcholine receptors (AChRs) at the NMJ.Slides were mounted in paraphenylenediamine to inhibit quenching of fluorescence. Staining was visualized using fluorescein and rhodamine optics on a Zeiss Axiohot epifluorescence microscope or using a Zeiss LSM 700 or LSM510 confocal microscope.
2.2.4. Quantitation of muscle morphometry, neuromuscular diameter, and synaptic density

NMJ and myofiber cross-sectional area, number of AChR microaggregates, and central nuclei measurements were carried out using Zeiss AxioVision Rel.4.8 software. Neuromuscular diameter was measured using staining of NMJs with rhodamine-α-bungarotoxin in the tibialis anterior and/or gastrocnemius muscle cut in cross-section.

2.2.5. Infection of skeletal muscle with AAV1-CMV-Galgt1

Adeno associated virus, serotype 1 (AAV1), vector was synthesized and highly purified in the Viral Vector Core Laboratory at The Research Institute at Nationwide Children’s Hospital as previously described (Martin et al., 2009). Mouse Galgt1 cDNA was expressed in AAV1 vector using cytomegalovirus (CMV) promoter. Left gastrocnemius and tibialis anterior (TA) were injected intramuscularly with 25μL and 50μL of 1x10^{11} vg and 5x10^{11} vector genomes (vg) of AAV1-CMV-Galgt1 virus respectively in 7-day old WT pups, while the contralateral (right) side was mock-infected by injecting with an equivalent volume of PBS. The mice were sacrificed 6-week and 12-week post-viral injections. The mock-infected (right) and injected (left) muscles were dissected and snap-frozen in liquid nitrogen cooled 2-methylbutane.
2.2.6. Statistics

All determinations of significance were done by ANOVA with post-hoc Bonferroni analysis and/or unpaired t tests.

2.3. Results

2.3.1. Galgt1 and Galgt2 contribute in unique ways to the formation of synaptic CT carbohydrates at the neuromuscular junction.

We had previously made affinity purified polyclonal antibodies to Galgt2 to demonstrate that its protein expression is highly concentrated at the neuromuscular junction (NMJ) in skeletal muscle (Hoyte et al., 2002; Xia et al., 2002). Here, we made affinity purified antibody to Galgt1 and showed that it too was concentrated in neuromuscular regions of adult skeletal muscles (Figure 2.1). Galgt1 expression was highly co-localized with postsynaptic nicotinic acetylcholine receptors (AChRs), which were stained with α bungarotoxin. Thus Galgt1, like Galgt2, likely synthesizes βGalNAc-linked carbohydrate antigens locally at the synapse due to its concentration in synaptic regions of skeletal myofibers.

Because both Galgt1 and Galgt2 are concentrated in postsynaptic regions of skeletal muscles, we took a genetic approach to understanding their relative contributions to synaptic expression of pre- and postsynaptic CT carbohydrates. To do this, we analyzed CT1 and CT2 staining at the NMJ in mice containing genetic deletions
of Galgt1 (Galgt1-/-), Galgt2 (Galgt2-/-) or both Galgt1 and Galgt2 (Galgt1-/-Galgt2-/-). All three genetic deletion strains were compared to wild type (WT) animals maintained on a similarly pure genetic background (C57Bl/6). In WT muscle, CT1 expression was often offset from α bungarotoxin staining, consistent with presynaptic localization, while CT2 expression was more coincident with postsynaptic AChR expression, much as we had previously observed (Martin et al., 1999), as well as at myotendinous junctions (Figure 2.2). In Galgt1-/- muscle, presynaptic CT1 expression was lost, while CT2 expression was maintained on and near the postsynaptic membrane. Some extrasynaptic expression of CT2 was also present in Galgt1-/- muscle that was not obvious in WT muscle. This staining may reflect altered CT expression due to loss of Galgt1, but CT2 staining was nevertheless still present at NMJs. In Galgt2-/- muscles, CT1 expression remained present at NMJs in a presynaptic pattern, while postsynaptic CT2 expression was lost (Figure 2.2). Intracellular and juxtamembrane puncta of CT1 immunostaining were also present in Galgt2-/- muscles. In Galgt1-/-Galgt2-/- muscle, staining of both pre and postsynaptic CT carbohydrate was absent (Figure 2.2). Only non-specific staining of large blood vessels was evident in these muscles. These data demonstrate that Galgt1 and Galgt2 both contribute to synaptic expression of the CT carbohydrates, with Galgt1 being required for proper presynaptic expression and Galgt2 for proper postsynaptic expression.
Figure 2.1. Galgt1 is expressed at the neuromuscular junction. An affinity purified Galgt1 antisera was used to immunostain wild type skeletal muscle (Gastroc), which was co-stained with rhodamine-α-bungarotoxin to label nicotinic acetylcholine receptors (AChR) concentrated at the neuromuscular junction (NMJ). Staining was compared to control staining with secondary antibody alone (2nd Only). Bar is 10µm for all images.
Figure 2.2. Galgt1 and Galgt2 both contribute to expression of the CT carbohydrate at the neuromuscular junction. Skeletal muscle (Tibialis anterior) from 6 week-old wild type (WT), Galgt1−/−, Galgt2−/−, and Galgt1−/−-Galgt2−/− was stained with CT1, CT2, or secondary antibody only (green) and with rhodamine-α-bungarotoxin, to label acetylcholine receptors (AChR) at the NMJ (red). Bar is 20µm for all images.
2.3.2. Dystroglycan is required for postsynaptic localization of CT2 at the neuromuscular junction.

Overexpression of Galgt2 in skeletal muscle leads to increased glycosylation of \( \alpha \) dystroglycan with the CT2 carbohydrate, and \( \alpha \) dystroglycan is the predominant glycoprotein evident in Galgt2 transgenic muscles (Nguyen et al., 2002; Xia et al., 2002). Dystroglycan (both the \( \alpha \) and \( \beta \) chain) is present along the entirety of the sarcolemmal membrane, but is also concentrated in the postsynaptic membrane at the NMJ (Matsumura et al., 1992), where Galgt2 and CT2 are also normally localized (Martin et al., 1999; Xia et al., 2002). Because dystroglycan is an essential gene in mice (Williamson et al., 1997) and because deletion of dystroglycan in skeletal muscles causes muscular dystrophy (Cohn et al., 2002), we utilized a unique Pax3-driven Cre transgenic line to delete a floxed allele of dystroglycan (\( Dag1^{loxP/loxP} \)) specifically in hindlimb skeletal muscles (Jarad and Miner, 2009) to assess dystroglycan’s role in postsynaptic CT2 expression.

We first confirmed that P3Pro-CreTg\( Dag1^{loxP/loxP} \) mice had no expression of \( \alpha \) or \( \beta \) dystroglycan in skeletal myofibers of hindlimb muscles, including the gastrocnemius and tibialis anterior (TA) (Figure 2.3). Dystroglycan was also absent from NMJs in these muscles, as evidenced by lack of co-staining with rhodamine-\( \alpha \)-bungarotoxin (Figure 2.3). Because deletion of dystroglycan causes muscular dystrophy, there was expression of dystroglycan in some mononuclear cells within the muscle, most likely macrophages,
however expression was absent in skeletal myofibers. By contrast, dystroglycan was expressed normally in forelimb muscles such as the triceps and biceps of P3ProCreDag1\textsuperscript{loxP/loxP} mice, due to lack of Cre expression in these muscles (Jarad and Miner, 2009) (Figure 2.3).

In the gastrocnemius muscle of P3Pro-CreDag\textsuperscript{1loxP/loxP} mice, which is Dag1-deleted (Jarad and Miner, 2009), CT1, which is normally expressed in the presynaptic membrane (Martin et al., 1999), was still present (Figure 2.4). By contrast, CT2 staining, which is normally postsynaptic (Martin et al., 1999), showed reduced or absent colocalization with AChRs-rich domains at NMJs (Figure 2.4). Instead, CT2 staining primarily was present in intracellular puncta, with occasional patches of sarcolemmal membrane staining that was removed from AChRs. All muscles showed some CT carbohydrate staining of intramuscular capillaries, as previously described (Martin et al., 1999), which were evident as bright puncta separate from NMJs (Figure 2.4). There was some increase in Galgt2 and CT2 expression in certain Dag1\textsuperscript{−/−} myofibers due to the dystrophic conditions of these dystroglycan-deficient muscles, but CT2 expression was never coincident with AChRs in such muscles. CT2 staining was, however, coincident with AChRs in Dag1-expressing hindlimb muscles taken from wild type, P3Pro-Cre or Dag1\textsuperscript{loxP/loxP} mice (Figure 2.4). Therefore, dystroglycan is required for the proper localization of postsynaptic CT2 carbohydrate at the NMJ.
Figure 2.3. Deletion of dystroglycan expression in P3ProCre\textit{Dag1}\textsuperscript{loxP/loxP} hindlimb muscles. Skeletal muscles from P3Pro-Cre\textit{Dag1}\textsuperscript{loxP/loxP} mice were co-stained with an antibody to β dystroglycan (A) or α dystroglycan (B) (green) and rhodamine-α-bungarotoxin (red) to label AChRs at NMJs. Merged images are shown, with overlap as yellow. Some NMJs are marked with an asterisk. Some mononuclear cells expressing α and β dystroglycan in dystrophic \textit{Dag1}-deleted muscles are marked with an arrow. Bar is 50\,\mu m for all panels. TA, Tibialis anterior
Figure 2.4. Synaptic expression of the CT2 carbohydrate is altered in Dag1⁻/⁻ muscle. Skeletal muscles from 8 week-old P3ProCreDag1⁺/-lox/lox mice (Dag1⁻/⁻, Gastrocnemius) were stained with CT1, CT2 or secondary antibody alone (2nd Only) (green) and co-stained with rhodamine-α-bungarotoxin (red) to label acetylcholine receptors (AChR) at the NMJ. Control wild type (Dag1⁺/⁺, Quadriceps) muscle was stained with CT2 and rhodamine-α-bungarotoxin. Bar is 25µm for all panels.
2.3.3. Transgenic overexpression of Galgt1 in skeletal muscle leads to muscle pathology.

To assess the effects of Galgt1 overexpression in skeletal muscle, we infected muscles with AAV1-CMV-Galgt1, an adeno-associated virus (AAV) vector designed to overexpress the Galgt1 cDNA. We injected AAV-CMV-Galgt1 into wild type Gastroc and TA muscles at one week of age, and analyzed Galgt1 protein and CT carbohydrate antigen expression at 6 or 12 weeks post-infection. Single stranded AAV vectors typically take 3 weeks to reach maximal gene expression in skeletal muscle and largely only infect skeletal myofibers when injected in this manner (Xiao et al., 1998; Xu et al., 2007). By 6 weeks post-infection, we identified large numbers of myofibers overexpressing Galgt1 protein in an intracellular pattern consistent with its normal localization to the Golgi apparatus (Figure 2.5A). Unlike overexpression of Galgt2, however, which leads to uniform distribution of CT carbohydrate along the sarcolemmal membrane (Nguyen et al., 2002; Xia et al., 2002), Galgt1 overexpression only led to accumulation of intensely stained intracellular and juxtamembrane punta using anti-CT carbohydrate antibodies (Figure 2.5A). By 6 weeks of age, Galgt1 overexpression caused a reduction skeletal muscle growth; Average myofiber diameters were significantly reduced in AAV1-CMV-Galgt1-infected muscles (Figure 2.5B, by 22% in the Gastroc (P<0.001) and by 17% in the TA (P<0.05)). This reduction was retained at 12 weeks post-infection (16% in Gastroc, P<0.01 and 21% in TA, P<0.05). By 12 weeks post-infection, Galgt1 overexpression had induced a significant increase in the number of myofibers with central nuclei (Figure
2.5C) and in the variance of myofiber diameters (Figure 2.5D). Both of these measures are indicative of increased muscle pathology. Thus, Galgt1 overexpression was unable to induce significant membrane expression of the CT carbohydrate and instead stimulated intracellular accumulation within skeletal myofibers associated with reduced muscle growth and increased muscle pathology. One additional unusual feature of Galgt1 muscle overexpression was the accumulation of mononuclear cells near large blood vessels (Figure 2.5B, lower left panels), which was noted on multiple occasions.
Figure 2.5. Galgt1 overexpression in skeletal myofibers fails to induce membrane expression of CT carbohydrate and causes myopathic changes. (A) Galgt1 protein and CT carbohydrate immunostaining (CT2) of an AAV1-CMV-Galgt1-infected wild type muscle at 6 weeks post-infection. Bar is 50µm for all panels. (B) Hematoxylin and eosin staining of cross-sections of Gastrocnemius (Gastroc) and Tibialis anterior (TA) muscles at 6 weeks or 12 weeks post-infection of AAV1-CMV-Galgt1 compared to mock-infected contralateral muscle control muscles taken from the same animal. Bar is 50µm for all panels. (C) Quantification of the percentage of myofibers with centrally located nuclei in AAV1-CMV-Galgt1-infected muscles and mock-infected contralateral controls. n=6-15 muscles per condition (D) Quantification of the variance in myofiber diameter. Measures below 250 (line) are considered normal, while higher measures are suggestive of dystrophy. n=6-15 muscles per condition. Errors are SEM. ***P<0.001.
2.3.4. A neuromuscular phenotype in adult Galgt2-/- mice skeletal muscles is seen with instability of acetylcholine receptors.

Galgt2-deficient mice (Galgt2-/-) have no overt neuromuscular phenotypes such as weakness or spasticity that would suggest dramatic alterations in NMJ biology. Analysis of Galgt2-/- muscles, however, showed increased accumulation of α bungarotoxin staining in intracellular microaggregates by 3 months of age (Figure 2.6). Staining for synaptic α dystroglycan and synaptic dystroglycan-associated proteins (utrophin) and ligands (laminin α5, agrin) showed no change in Galgt2-/- muscle relative to wild type, and at 6 weeks of age no appreciable intracellular accumulations of AChRs were evident (Figure 2.6). Staining of laminin α2 and α sarcoglycan, which are present in both extrasynaptic and synaptic regions, was also normal (Figure 2.6)

In muscles from mice of 3 months or older, intracellular micro-aggregates of AChRs were present and these co-localized with acetylcholinesterase, a βGalNAc-containing synaptic cleft glycoprotein (Figure 2.6) (Scott et al., 1988). 3 month-old Galgt2-/- gastrocnemius muscle had an order of magnitude increase in intramuscular AChR microaggregates compared to age-matched wild type muscle (Figure 2.6)

The cross sectional area of the NMJ and of skeletal myofibers, however, was unchanged in Galgt2-/- animals (Figure 2.6). While a subtle phenotype, these studies demonstrate that Galgt2-deficiency alters the patterning of synaptic AChR expression with age and may reflect their changed synaptic stability.
To assess what intracellular compartments AChR microaggregates might be in, we co-stained muscle from 3 month-old Galgt2-/- mice for AChRs and markers for the endoplasmic reticulum (ER, calnexin), trans-Golgi (TGN38), lysosome (Lamp1) and endosome (clathrin heavy chain) (Figure 2.7). AChR microaggregates did not co-stain with Lamp1 or calnexin, suggesting no trapping of intracellular AChRs in the ER or lysosome. A few AChR microaggregates were co-localized with the trans-Golgi marker TGN38, but many more co-stained with clathrin heavy chain, demonstrating increased expression of AChRs in endosomes. No such increase in intracellular aggregates was observed in Galgt1-/- muscles (ns), consistent with previously published data (Bullens et al., 2002). Thus, Galgt2-deficiency appears to alter AChR dynamics such that AChRs are increasingly internalized via endosomes with age or may also be trapped in the trans-Golgi or in endosomes prior to secretion.
Figure 2.6. Neuromuscular phenotype in Galgt2-/- muscle. (A) Galgt2-/- skeletal muscle cross-sections taken from mice at 6 weeks of age were immunostained with antibodies to α dystroglycan (αDG), α sarcoglycan (αSG), utrophin, agrin, laminin α2 (LNα2), or laminin α5 (LNα5) (green). Sections were co-stained with rhodamine-α-bungarotoxin to label nicotinic acetylcholine receptors (AChRs) at the NMJ (red). Bar is 50µm. (B) High power images of AChRs co-labeled with acetylcholinesterase (AChE) in Galgt2-/- muscles at 3 months of age. Bar is 10µm. Intracellular muscle AChR microaggregates (C), average cross-sectional NMJ area (D) and average cross-sectional myofiber area (E) at 3 months of age were compared in wild type (WT) and Galgt2-/- muscles. n=37 (Galgt2-/-) or 46 (WT) synapses per condition in C and D and n=110 (Galgt2-/-) or 140 myofibers per condition in E for at least 6 animals per measure. Errors are SEM for all figures.
Figure 2.7. Co-localization of intracellular AChR microaggregates with an endosomal marker in Galgt2/-
muscle. Skeletal muscle cross-sections from 3 month-old Galgt2/- animals were co-stained with
calnexin, clathrin heavy chain, LAMP1, or TGN38 and rhodamine-α-bungarotoxin to label AChRs. Arrows
mark representative regions of α bungarotoxin-positive AChR microaggregates in both panels. Bar is 50µm.
2.4. Discussion

The fact that the same carbohydrate structure can be made by different glycosyltransferases on different classes of molecules significantly complicates the understanding of carbohydrate function. This challenge is made even more difficult with regard to understanding the roles of carbohydrates at synapses, because glycoproteins and glycolipids enriched there may emanate from multiple cellular elements. For the neuromuscular junction (NMJ), these cellular players include the presynaptic motor neuron, the postsynaptic skeletal myofiber, and the pre-synaptic non-myelinating Schwann cell (Sanes and Lichtman, 2001). As a group, these cells contribute to the synaptic concentration of β-linked terminal N-acetyl-D-galactosamine (βGalNAc) in species ranging from lampreys to humans (Hoyte et al., 2002; Martin et al., 1999; Sanes and Cheney, 1982; Scott et al., 1988).

Here we describe the concentration of two β1,4GalNAc glycosyltransferases, Galgt1 and Galgt2, in synaptic regions of mouse skeletal myofibers. The postsynaptic concentration of both enzymes is consistent with the finding that antibodies to the CT carbohydrate, which can reflect glycoproteins, and antibodies to GD2, a βGalNAc-terminated ganglioside, both stain the postsynaptic muscle membrane (Martin et al., 1999). Through analysis of mice lacking Galgt1 and/or Galgt2 by gene deletion, we show that Galgt1 is required for presynaptic βGalNAc expression of the CT carbohydrates and Galgt2 for postsynaptic expression. This does not discount the possibility that other
βGalNAc transferases, for example those involved in the biosynthesis of chondroitin sulfate glycosaminoglycans, may not also be present and contribute to synaptic glycosylation in ways we have not detected here. In addition, Galgt1-/- muscle did show some extrasynaptic muscle expression of CT2. As such, postsynaptic gangliosides may have a role, though not an absolute one, in facilitating or consolidating postsynaptic CT carbohydrate expression. In addition, post-synaptic concentration of Galgt1 activity may be masked by subsequent modification of GM2 or GD2 on β1,4GalNAc, for example to make GM1, which we have shown is also localized at the NMJ. A simplistic model emerging from this genetic data, however, is that presynaptic CT carbohydrates occur predominantly on gangliosides, while postsynaptic CT carbohydrates occur on Galgt2-dependent glycoproteins and non-GM2/GD2 glycolipids. Indeed, overexpression of Galgt2 in skeletal muscle leads to increased CT glycosylation of α dystroglycan and at least one non-GM2/GD2 glycolipid, which supports such a model (Nguyen et al., 2002; Xia et al., 2002; Xu et al., 2007).

We also describe here a role for dystroglycan in localizing the CT carbohydrates at the NMJ. While we cannot yet ascertain the relative contributions of particular Galgt2 substrates to overall postsynaptic CT carbohydrate expression, elimination of dystroglycan from skeletal myofibers led to a failure of CT carbohydrate to localize properly to the postsynaptic muscle membrane. Thus, dystroglycan is required for the proper postsynaptic expression of the CT carbohydrate and may therefore be a synaptic organizer of Galgt2 activity or perhaps a synaptic Galgt2 substrate. α dystroglycan is
clearly a substrate for Galgt2 *in vitro* (Yoon et al., 2009). Overexpression of Galgt1 in skeletal muscle, by contrast, did not allow for membrane expression of CT carbohydrate and instead caused muscle pathology. This may be due to a lack of ganglioside substrates for Galgt1 in the extrasynaptic muscle membrane or an inability of GM2 or GD2, once made, to maintain membrane expression. Regardless, the presence of increased CT carbohydrate staining in intracellular puncta after Galgt1 overexpression suggests Galgt1 is unable to accomplish the dramatic membrane overexpression of CT carbohydrate that can occur with Galgt2, which can, in turn, alter the expression of synaptic muscle molecules and inhibit disease in at least three mouse models of muscular dystrophy (Martin et al., 2009; Nguyen et al., 2002; Xia et al., 2002; Xu et al., 2007; Xu et al., 2009).

Given that cell surface carbohydrates are also receptors for infectious agents, particularly viruses and bacteria, and thus often have altered tissue expression during the course of evolution (Varki, 2006), the phylogenetic conservation of βGalNAc at the vertebrate NMJ suggests this carbohydrate has important functions there. Elimination of both Galgt1 and Galgt2 in mice, however, argues to the contrary, as these animals move and breathe normally for many months. Previous studies have shown that deletion of Galgt1 in mice leads to Wallerian degeneration of motor axons in aging animals (Pan et al., 2005; Sheikh et al., 1999). This only occurs once the mice are well into adulthood, beginning at 6 months of age (Pan et al., 2005; Sheikh et al., 1999). Prior to that, NMJs in Galgt1-deleted mice show no obvious change in NMJ structure or function, aside from
losing pre-synaptic binding to botulinum toxin (Bullens et al., 2002). All of our studies have focused on early postnatal development, up to 3 months, so as to bypass issues that might result from loss of Galgt1 in aging animals. In this early time period, loss of Galgt2 causes apparent neuromuscular instability, as evidenced by increased expression of acetylcholine receptors within skeletal myofibrer, some in clathrin-coated endosomes. Given that Galgt2 overexpression causes increased CT glycosylation of α dystroglycan (Nguyen et al., 2002; Xia et al., 2002), stimulates ectopic overexpression of synaptic dystroglycan binding proteins (such as laminins α4, laminin α5 and agrin (Xia and Martin, 2002; Xu et al., 2007)), and increases laminin and agrin binding to dystroglycan (Yoon et al., 2009), and given that synaptic laminins, agrin, and dystroglycan are required for neuromuscular development, stability and/or maturation (Burgess et al., 1999; Cote et al., 1999; Gautam et al., 1996; Misgeld et al., 2005; Nishimune et al., 2008), it is tempting to hypothesize that loss of Galgt2 in mice inhibits CT glycosylation of α dystroglycan at the NMJ, weakening synaptic ECM-postsynaptic membrane integrity and/or signaling, thereby stimulating AChR membrane turnover. Future work will be required to test this theory. Acetylcholinesterase is also glycosylated with βGalNAc, on its synaptic ColQ subunit, in mouse skeletal muscle (Scott et al., 1988) and shows increased expression in intracellular aggregates at the NMJ in Galgt2-/- mice. Therefore, loss of βGalNAc glycosylation on acetylcholinesterase could also be mediating such changes.
CHAPTER 3. Role of Galgt1 in skeletal muscle regeneration.

3.1. Introduction.

The ability of skeletal muscle to regenerate itself is critical for its repair and maintenance. The capacity of the skeletal muscle to regenerate is due, in large part, to satellite cells. Satellite cells are the myogenic stem cell population that resides along the myofiber beneath the basal lamina. They get activated in response to injury or stretch stimuli. Satellite cells then undergo asymmetric division to self-renew and to differentiate into myoblasts. As myoblasts are generated, they also migrate along the basal lamina within the region of damage and ultimately fill this space and fuse with one another to make a new myofiber. Disruption or defect in any one of these processes will lead to insufficient muscle regeneration. In the muscular dystrophies, such impaired regeneration often leads to muscle wasting (loss of muscle tissue and replacement with extracellular matrix or fat), which drives muscle weakness and disease severity. These processes are probably mediated by signaling molecules present on the cell surface. Glycolipids are good candidates for these processes. It has been recently shown that
clustering and dispersal of lipid rafts at the lamelliopodia of myoblasts helps in membrane fusion (Mukai et al., 2009). GM1, a complex ganglioside and a subsequent product of Galgt1, is localized in these lipid rafts and binding of GM1 to cholera toxin subunit β enhances myoblast membrane fusion (Mukai et al., 2009). In this study, we investigate the role of Galgt1 and GM1 in skeletal muscle regeneration. We found that Galgt1 and GM1 both are overexpressed in satellite cells following acute or chronic muscle injury and that in absence of Galgt1, skeletal muscle regeneration is delayed.

3.2. Materials and Methods

3.2.1. Materials

Anti-ganglioside GM1 antibody was bought from Millipore (345757). Rabbit polyclonal antibody to Galgt1 peptide CQVRAVDLTKAFDAE was made by immunizing rabbits with KLH-conjugated peptide, after which antibody was purified over peptide-conjugated resin as previously described (Xia et al., 2002). Anti-mouse integrin α7 conjugated to FITC was bought from MBL International (K0046-4). Anti-mouse integrin α7 conjugated to FITC was purchased from R&D Systems (FAB3518F). Rat anti-Ertr7 was gift from Dr. Montanaro. Mouse anti-CD11b conjugated to FITC purchased from Abcam. Rat anti-mouse Ly-6A/E conjugated to FITC (Sca1, 553335), rat anti-mouse CD45 conjugated to PE-Cy7 (552848), rat anti-mouse CD31 conjugated to APC (551262) and rat anti-mouse CD16/CD32 Fc block (553142) were bought from BD Biosciences. All secondary
antibodies conjugated to fluorophores were bought from Jackson ImmunoResearch. Rhodamine α bungarotoxin was purchased from Molecular Probes.

3.2.2. Mice

Mice lacking Galgt1-/- were obtained from Consortium for Functional Glycomics. mdx was bought from Jackson Laboratories. WT mice (C57BL/6) and were bought from Jackson Laboratories. Galgt1-/-mdx mice were obtained by interbreeding of Galgt1-/-mice with mdx mice. 2 month old animals were used for cardiotoxin experiment and histology. 6 weeks (young) and 6 months (old) animals were used for WT, Galgt1-/-, mdx and Galgt1-/-mdx experiments.

3.2.3. Cardiotoxin Intramuscular Injection

Cardiotoxin, a toxin from Naja mossambica mossambica’s venom, was purchased from Sigma-Aldrich (C9759). It was diluted to 10µM in 1x phosphate buffered saline (PBS) and injected intramuscularly into the gastrocnemius, tibialis anterior and/or quadriceps muscles in the left hindlimb. The right hindlimb was injected with 1x PBS as a control. Muscles were collected 1, 4, 7, 14 and 28 days after cardiotoxin injection for acute injury muscle regeneration experiments.

3.2.4. Histology

Muscles were dissected, mounted on OCT and snap frozen in liquid nitrogen cooled 2-methylbutane. The muscles were cross-sectioned on cryostat at 8 µm. For
immunostaining, the sections were blocked in 3mg/ml BSA in 1x PBS. The primary antibodies were diluted in 3mg/ml BSA/PBS and sections incubated with it overnight at 4°C. The sections were stained with secondary antibody, added at a concentration of 1:250, for 1 hour. The slides were mounted in Prolong Gold Antifade from Invitrogen. For hematoxylin and eosin (H&E) staining the sections were first fixed in 10% neutral buffered formalin, washed in tap water, stained in Gill’s 3 Hematoxylin for 2 mins, washed in tap water, then bluing agent and again tap water. Sections were then stained in Eosin for 1 min and excess stain removed by dipping in 30% ethanol, after which the sections were dehydrated in 70%, 90%, 95%, and 100% ethanol. The sections were cleared in xylene and mounted in a xylene based mounting media, Cytoseal. Staining was imagined on Zeiss Axiophot epifluorescence microscope.

### 3.2.5. Creatine Kinase Assay

100-200µL blood was collected and clotted to separate out the serum. The serum was either diluted or used undiluted for the assay. Diagnostic chemicals limited’s creatine kinase SL assay kit (326-10) was used to quantify the creatine kinase (CK) levels. Manufacturer’s instructions were followed to measure the serum CK enzyme activity using a Thermo Electron Corporation’s Genesys 10 UV Scanning spectrophotometer.
3.2.6. Quantitation of Muscle Morphometric Measurements

Myofiber diameter and central nuclei measurements were carried out using Zeiss AxioVision Rel.4.8 software. H&E images were used for measurements at 20x. Five sections were analyzed per animal, with 4 animals per time point per genotype.

3.2.7. Fluorescence Activated Cell Sorting (FACS)

The left limb of three WT mice were injected with 10µM cardiotoxin and the right limb with 1x PBS. One day after the injection, the muscles were removed, minced and digested with 5mg/ml collagenase IV and 1.2U/ml dispase for 30 minutes at 37°C. The reaction was stopped by addition of 1x DMEM and the cells were filtered through a 70µm membrane, followed by filtration on a 40µm cell strainer. Cells were then centrifuged in horse serum and stained after washing in 0.5%BSA/PBS. The cells were blocked with rat anti-mouse CD16/CD32 (Mouse BD Fc Block, BD Pharmingen 553141) at a concentration of 2µl/10⁶ cells on ice for 10 minutes; followed by staining with fluorophore-conjugated primary antibody staining at a concentration of 2µl/10⁶ cells for 10 mins on ice. Cells were the washed and resuspended and filtered in FACS tubes. 2µg/ml propidium iodide (PI) was added just before sorting to identify dead cells. Cells were first gated based on side scatter and forward scatter then based on the height and width. Dead cells take up PI and were removed prior to further analysis. Cells were then sorted for Sca1+ and Sca1- fractions and each fraction was further sorted for CD31+, CD45+ and CD31-CD45-. Sca1+CD31+CD45-, Sca1+CD31-CD45-, Sca1-CD31+CD45+, Sca1-CD31-CD45-, Sca1-
CD31-CD45- fractions were collected and analyzed for Galgt1 mRNA using qRT-PCR.

FACS was repeated with gating for CD31-APC and CD45-PE Cy7 and the CD31-CD45- fractions, with sorting for integrin α7 against side scatter. The integrin α7+CD31-CD45-, integrin α7-CD31-CD45-, CD31+CD45-, and CD31-CD45+ fractions were collected and analyzed for Galgt1 mRNA using qRT-PCR.

3.2.8. Semi-quantitative Real-time PCR (qRT-PCR)

Gastrocnemius muscles were homogenized in Trizol (Invitrogen) and total RNA was extracted and purified using either RNeasy Mini or Micro kits (Qiagen) according to the manufacturer’s instructions. The quality of RNA was determined by electrophoresis using 6000 Nano LabChip kit on Bioanalyzer 2100 (Agilent). The samples without RNA degradation were used to synthesize cDNA using High Capacity Reverse Transcriptase kit (Applied Biosystems). Taqman ABI 7500 sequence detection system (Applied Biosystems) was used for real-time PCR measurements on the samples. GAPDH was used as an internal control. The forward primer sequence used for Galgt1 was GAGCTACCAGGCCAACACA and the reverse primer sequence was GGCAACTTCATGTCCCTTGGT and the sequence probe was AACCGGACTGTGTCTGC.

3.2.9. Statistics

All determinations of significance were done by ANOVA with post-hoc Bonferroni analysis and/or unpaired t tests. A p-value of less than or equal to 0.05 was considered significant.
3.3. Results

Skeletal muscle has an enormous and quite remarkable capacity to regenerate after injury. Skeletal muscle regenerates in response to injury caused by daily wear and tear as well as trauma and/or disease. Muscle regeneration can be studied either in chronic or an acute injury model. Dystrophic muscle such as that of mdx mice provides an opportunity to study chronic regeneration, with the frailty of the muscle membrane giving rise to continuous cycles of muscle degeneration and regeneration. There are several ways to study acute regeneration. Most common acute injury model is to inject muscles with cardiotoxin (CTX, *Naja mossambica mossambica*). In this study, we have used both acute and chronic regeneration to look at the role of Galgt1 in skeletal muscle regeneration.

3.3.1. Galgt1 is highly upregulated in a regenerating skeletal muscle after cardiotoxin-induced muscle damage.

We originally queried Dr. Eric Hoffman's public Affymetrix microarray data on cardiotoxin-induced muscle regeneration in wild type muscles [http://microarray.cnmcresearch.org](http://microarray.cnmcresearch.org), now [http://pepr.cnmcresearch.org](http://pepr.cnmcresearch.org), looking at the 1769 glycosylation-related genes listed on the glycan microarray from the Consortium for Functional Glycomics ([http://www.functionalglycomics.org](http://www.functionalglycomics.org)). Of these genes, Galgt1 was the most elevated glycosylation gene relative to control, with a 56- to 64-fold...
increase in signal, in first two days after cardiotoxin treatment that subsided back to near baseline levels by 16 days post-treatment (Figure 3.1).

Figure 3.1. Microarray data showing multi-fold increase in Galgt1 signal after cardiotoxin injection of skeletal muscle.

To validate the findings from the microarray data, we injected WT mouse muscle with CTX, collected the injected muscles at various time-points (days 0, 1, 4, 7, 14, and 28),
and extracted mRNA for qRT-PCR analysis. We confirmed the microarray data and found that Galgt1 mRNA was significantly upregulated, by 23-fold, on day 1 after cardiotoxin injection. On day 4, the upregulation of Galgt1 mRNA decreased to 10-fold elevation and on day 7 to 7-fold, but this was still significantly upregulated as compared to contralateral mock-injected control muscles. On day 14 and day 28, Galgt1 levels dropped to near normal levels, 2.6- and 1.8-fold respectively (Figure 3.2).
Figure 3.2. Fold change in the expression of Galgt1 gene in WT muscle after cardiotoxin injection. After Day1 of the injury, the expression of Galgt1 gene increases 23-fold when compared to an mock-injected muscle (Day0). The expression of Galgt1 reduces as the muscle regenerates and reaches near normal levels on Day28. Errors are SEM.
To see if this enormous increase in Galgt1 mRNA was translated to increase in protein expression, we stained the muscle cross-sections from gastrocnemius (gastroc) or tibialis anterior (TA) with Galgt1 antibody, and GM1 ganglioside antibody. GM1 is a complex ganglioside that requires Galgt1 activity and does not cross react with CT antigen, as antibodies to GM2 ganglioside do. Galgt1 and GM1 both stained the neuromuscular junction at day 0 in mock-injected muscle. To our surprise, Galgt1 and GM1 were both highly upregulated in mononuclear cells on day 1. This staining of mononuclear cells was still present on day 4 but the staining was also observed in and around the newly formed immature myofibers. By day 7 the staining had reduced and was present around a small number of myofibers and by day 14 and day 28, it was again
localized to the neuromuscular junction similar to the mock-transfected day 0 staining (Figure 3.3).
Figure 3.3. Different stages of regeneration in WT Gastocnemius muscle injected intramuscularly with cardiotoxin (CTX). Days are indicative of the number of days after cardiotoxin injection. Day 0 is the uninjected muscle. GM1 antibody stains the WT muscle at Day 0, this staining increases considerable on Day 1 after CTX injection. The staining is present in and around the mononuclear cells and muscle fibers. On Day 4 the staining is seen around the newly regenerated muscle fibers. The staining reduces thereafter and is reaches normal level on Day 14 and Day 28, by which time the muscle has fully regenerated. Galgt1 antibody stains the NMJ on Day 0. After Day 1 of injury, the staining is elevated in mononuclear cells. On Day 4 the staining is still present in the mononuclear cells but is also around the newly regenerated muscle fibers. Galgt1 staining is reduced after Day 4 and is present only at NMJ on Day 14 and Day 28 as the muscle returns to normal after injury. The secondary antibody (anti-rabbit IgG) control is blank therefore the staining seen is specific to the primary antibody. The micron bar is 50µm.
3.3.2. Galgt1 and GM1 are localized at the neuromuscular junction in wild type skeletal muscle and in mononuclear cells in an injured muscle.

In a normal uninjured muscle, Galgt1 is localized at the NMJ (Figure 2.1, Figure 3.4). To determine the expression pattern of GM1, the subsequent product of Galgt1, we stained WT gastrocnemius with GM1 antibody. GM1, like Galgt1, was also localized at the NMJ, which was visualized by co-staining the muscle sections with α-bungarotoxin (BTX, Figure 3.4). GM1 staining was also present elsewhere within skeletal muscle sections, likely in intramuscular capillaries.
Figure 3.4. Staining of the neuromuscular junction (NMJ) with GM1 and Galgt1 antibodies in WT and Galgt1-/- mice. Neuromuscular junctions are visualized by co-staining with bungarotoxin (BTX, in red channel). GM1 and Galgt1 antibody staining is seen in green channel. In WT mice, both antibodies stain the neuromuscular junctions and this staining is absent from the Galgt1-/- muscle. Therefore, the enzyme Galgt1 and the ganglioside GM1 both are present at the NMJ. The micron bar is µm. The micron bar is 50µm.
The expression pattern of both Galgt1 and GM1 changes with muscle disease. The expression of Galgt1 (Figure 3.5) and GM1 (Figure 3.6) increases in mononuclear cells and around the myofibers in mdx muscle, this expression pattern is similar to that seen during the acute muscle regeneration followed by cardiotoxin injury. Galgt1 mRNA is upregulated 13-fold in mdx mice (Martin Lab). The expression is, however, absent from both the Galgt1−/− and Galgt1−/−mdx muscles. This is to be expected as no complex ganglioside is synthesized in absence of Galgt1 enzyme.
Figure 3.5. Galgt1 antibody staining in WT, mdx, Galgt1-/-, and Galgt1-/-mdx muscle. Galgt1 staining is present at the NMJ in WT muscle. In mdx muscle, Galgt1 stains the satellite cells around the muscle fiber and in the regenerating regions of the muscle. This staining of satellite cells by Galgt1 antibody is absent in the Galgt1-/- and Galgt1-/-mdx muscle. The micron bar is 50µm.
Figure 3.6. GM1 ganglioside staining in WT, mdx, Galgt1-/-, and Galgt1-/-mdx muscle. GM1 antibody stained WT and mdx muscle. In mdx muscle, GM1 antibody pre-dominantly stained the regenerating regions and can be seen around the small fibers. The GM1 staining is absent in Galgt1-/- and Galgt1-/-mdx muscle. The micron bar is 50µm.
3.3.3. Absence of Galgt1 during acute and chronic skeletal muscle injury models leads to delayed muscle regeneration.

During both acute and chronic model of muscle, Galgt1 and GM1 expression are upregulated primarily in mononuclear cells. To see whether this massive upregulation of Galgt1 and its product GM1 following injury is important for some functional aspect of muscle regeneration, we studied muscle regeneration in absence of Galgt1. When gastrocnemius and TA muscles of Galgt1-/- mice were injected with cardiotoxin, muscle was still able to regenerate, showing that Galgt1 is not essential for skeletal muscle regeneration. However, we found several differences between WT and Galgt1-/- regenerating muscles. On Day 1 of CTX administration, there was a huge accumulation of mononuclear cells (See Figure 3.7) in Galgt1-/- muscles that was not typically seen in WT muscle. As muscle regenerated the number of mononuclear cells in WT muscle decreased but on Day 7 and Day 14, there were still many mononuclear cells present in the Galgt1-/- when compared to WT. The myofiber diameter of Galgt1-/- muscles seemed smaller than that of WT. So we quantitated the regenerated muscle fiber diameter on Day 14 and Day 28 muscles sections. The average myofiber diameter of Galgt1-/- muscles was significantly smaller than that of WT muscles on Day 14 (p-value < 0.05) (Figure 3.8). As a measure, average fiber diameter doesn’t always give a complete picture of muscle regeneration, as a regenerating muscle can have myofibers with different diameters and also with different distributions of myofiber diameter. So we plotted a distribution curve of muscle diameter to quantify changes in muscle size...
distribution in WT and Galgt1-/- mice after injury. The distribution curve for Galgt1-/- on Day 14 had shifted to the left when compared to WT, indicating that there were smaller fibers present in the Galgt1-/- when compared to WT (Figure 3.9). The average myofiber diameters of Galgt1-/- and WT mice on Day 28 after injury were not significantly different (Figure 3.10). These distribution curves, however, showed that there were still smaller muscle fibers present in Galgt1-/- as compared to WT (Figure 3.11). Although absence of Galgt1 didn’t completely abolished muscle regeneration, it did delay the rate at which the muscle regeneration occurred.
**Figure 3.7. Different stages of regeneration in WT and Galgt1-/-**: Gastrocnemius muscle injected intramuscularly with cardiotoxin (CTX). In Galgt1-/- muscle a great number of mononuclear cells are present at later stages of regeneration, whereas the WT muscle at later stages of regeneration looks more normal as most of the satellite cells have been fused. The micron bar is 50µm.
Figure 3.8. Average myofiber diameter of WT and Galgt1-/- gastrocnemius at Day 14 after cardiotoxin (CTX) injury. The average myofiber diameter of Galgt1-/- gastrocnemius at Day 14 after cardiotoxin injury is significantly smaller than that of WT. p-value<0.05. Errors are SEM.
Figure 3.9. Distribution of myofiber diameter of WT and Galgt1-/- gastrocnemius at Day 14 after cardiotoxin injury. The distribution of myofiber diameter of Galgt1-/- gastrocnemius at Day 14 after cardiotoxin injury is shifted towards left, that is there are more smaller fibers present, when compared to WT at same stage.
Figure 3.10. Average myofiber diameter of WT and Galgt1-/- gastrocnemius at Day 28 after cardiotoxin injection. There is no significant difference between the average myofiber diameter of WT and Galgt1-/- gastrocnemius at Day 14 of CTX injury. p-value=0.12. Errors are SEM.
Figure 3.11. Distribution of myofiber diameter of WT and Galgt1-/- gastrocnemius at Day 28 after cardiotoxin injury. Distribution of myofiber diameter of Galgt1-/- gastrocnemius at Day 28 of CTX injury is shifted to the left, that is there are smaller fibers present, when compared to WT inspite of no significant difference present in the average myofiber diameter.
To assess whether this delay in regeneration occurred with chronic injury, we interbred mdx and Galgt1-/- mice to obtain double knockout (Galgt1-/-mdx) mice. In young Galgt1-/-mdx mice, there was a marked increase in the area of necrosis when compared to mdx (Figure 3.12). The increase in the area of degenerating fibers suggested that regeneration was not taking place at the same rate or was not as effective in absence of Galgt1. These hyperproliferative regions are probably not due increase in damage caused by the loss of Galgt1, as in Galgt1-/- muscle histology looks normal (Figure 3.16). If the increase in area of damage is not caused by increased damage due to loss of Galgt1, then it is possible that it is due to a delay in regeneration. When we analyzed 6 month old mdx and Galgt1-/-mdx animals, we didn’t observe increased areas of necrosis resulting from loss of Galgt1. The average myofiber diameter of Galgt1-/-mdx was, however, significantly reduced as compared to mdx (p-value < 0.001) and the distribution curve was also shifted to the left, indicating that smaller fibers were present in Galgt1-/-mdx muscle as compared to mdx. The serum creatine kinase (CK) levels of both mdx and Galgt1-/-mdx were elevated, indicating muscle damage, as was expected. There was no significant difference found between the serum CK levels of WT and Galgt1-/- and between mdx and Galgt1-/-mdx. Therefore, loss of Galgt1 delays muscle regeneration kinetics and extent both in acute and chronic models of muscle regeneration.
Figure 3.12. H&E staining of Gastrocnemius in young (6 weeks) and old (6 months) mdx and Galgt1-/-mdx animals. Young (6 weeks old) Galgt1-/-mdx muscle shows marked hyperproliferative region with degenerating fibers and regenerated small muscle fibers indicative of impaired regeneration in Galgt1-/-mdx when compared to age-matched mdx. In older animals these regenerative areas are lesser in degree of severity. The micron bar is 50μm.
Figure 3.13. Average myofiber diameter of mdx and Galgt1-/-mdx gastrocnemius at 6-months. Average myofiber diameter of Galgt1-/-mdx gastrocnemius is significantly smaller than that of mdx, p-value<0.001. Errors are SEM.
The distribution of myofiber diameter of Galgt1−/−-mdx gastrocnemius at 6-months is shifted toward left, that is there are more smaller fibers present, when compared to age-matched mdx.
Figure 3.15. Average percentage central nuclei in mdx and Galgt1-/-mdx gastrocnemius at 6-months.

The average percentage of central nuclei in Galgt1-/-mdx gastrocnemius is significantly lower than the value in mdx. p-value<0.001. Errors are SEM.
Figure 3.16. Hematoxylin and eosin (H&E) staining of Gastrocnemius of WT, mdx, Galgt1-/- and Galgt1-/-mdx mice at 6-months. Galgt1-/-mdx muscle show more necrosis than mdx while WT and Galgt1-/- muscle looks normal. The micron bar is 50µm.
Figure 3.17. Creatine kinase (CK) assay at different time points of WT, Galgt1-/-, mdx, mdx/Galgt1-/-.

Creatine kinase activity was measured at 6 weeks, 3 months, and 6 months for WT, Galgt1-/-, and at 3 weeks, 6 weeks, 3 months, and 6 months mdx, Galgt1-/-mdx. No significant difference was observed between the CK values of WT and Galgt1-/- or mdx and Galgt1-/-mdx. No values are reported for WT and Galgt1-/- at 3 weeks. Errors are SEM.

3.3.4. Galgt1 is overexpressed in satellite cells following muscle injury.

Galgt1 mRNA is overexpressed greater than 10- fold in both acute and chronic injury. Since the loss of Galgt1 leads to delay in muscle regeneration and the upregulation is primarily seen in mononuclear cells. We wanted to find out what cell types were responsible for the dramatic increase in Galgt1 expression after injury. We used
Fluorescence Activated Cell Sorting (FACS) to answer this question. WT muscles, TA, quadriceps, and gastrocnemius, were injected with cardiotoxin and the muscles were processed the next day (Day 1) for FACS. We gated the cells for Sca1+ and Sca1-, we then sorted the Sca1+ and Sca1- populations for CD31+Sca1+ (collected), CD45+Sca1+, Sca1+CD31-CD45- (collected), CD31+Sca1-, CD45+Sca1- (collected) and Sca1-CD31-CD45- (collected) (Figure 3.18). Sca1 is a marker for mesenchymal stem cells, CD31 for endothelial cells, and CD45 for immune cells. The mRNA from various fractions was analyzed for Galgt1 upregulation by qRT-PCR. We found that Galgt1 mRNA was upregulated 11-fold in the Sac1-CD31-CD45- fraction (Figure 3.19). This fraction would contain satellite cells and fibroblasts. The Sca1+CD31-CD45- fraction showed an upregulation of 2.5-fold and there was no upregulation seen in other fractions. We then sorted again with integrin α7 to separate satellite cell population from endothelial (CD31), and immune cells (CD45). The integrin α7+CD31-CD45- population showed upregulation of Galgt1 mRNA by 4-fold (Figure 3.20). Whereas no upregulation of Galgt1 mRNA was observed in any other fraction collected. Since integrin α7 is a satellite cell marker (Sherwood et al., 2004), the FACS data suggested that Galgt1 is being upregulated in satellite cells. To verify this result, we double immunostained various muscle mononuclear cell markers, integrin α7 (satellite cell marker), CD11b (immune cell marker), Ertr7 (fibroblast marker), and Sca1 (mesenchymal cell marker) with GM1 and Galgt1 (Figure 3.21). We found similar results for both GM1 and Galgt1 (Data not shown for Galgt1). GM1 co-localized with integrin α7 (satellite cell marker) but did not
co-localize with Sca1 (mesenchymal stem cell marker), Ertr7 (fibroblast marker), and CD11b (immune cell marker). Similar results were obtained when 6 weeks old mdx gastrocnemius muscle was co-stained with GM1 and CD11b, and Ertr7. No staining for GM1 was observed in the Galgt1-/-mdx muscles (Figure 3.22, Figure 3.23, Figure 3.24). Therefore, our results show that Galgt1/GM1 upregulation seen in mononuclear cells are satellite cells, and that these cells predominantly contribute to the marked elevation of Galgt1 expression after injury.
Figure 3.18 Fluorescent activated cell sorting (FACS) of A. Cardiotoxin injected WT muscles and B. Non-injected WT muscles. A. The cardiotoxin injected Day 1 cells were first sorted for Sca1 marker against side scatter (SSC) and the both positive and negative populations were then sorted for CD31 and CD45. B. The control populations were sorted using the same gates as the cardiotoxin injected Day 1 cells. The percentages of cells present in each fraction are noted on the figure.
Figure 3.19 Real-time quantification of mRNA extracted from the Sca1 sorting. There was an upregulation of Galgt1 mRNA in Sca1-CD31-CD45- fraction by 11-fold when compared to the control fractions. Errors are fractional errors.
Figure 3.20 Real-time quantification of mRNA extracted from the integrin α7 sorting. There was an upregulation of Galgt1 mRNA in integrin α7+CD31-CD45- fraction by 4-fold when compared to the control fractions. Errors are fractional errors.
Figure 3.21  Double immunostaining of muscle mononuclear cell markers with GM1 in cardiotoxin injected Day 1 gastrocnemius. GM1 co-stains with integrin α7 but not with any other marker including CD11b, Ertr7, and Sca1. The micron bar is 50µm.
Figure 3.22 Double immunostaining of 6 weeks old mdx and Galgt1-/-mdx gastrocnemius with GM1 and integrin α7. GM1 co-stains with integrin α7 in mdx muscles but no GM1 staining was observed in Galgt1-/-mdx. The micron bar is 50μm.
Figure 3.23  Double immunostaining of 6 weeks old mdx and Galgt1-/-mdx gastrocnemius with GM1 and CD11b. GM1 does not co-stain with CD11b in mdx muscles and no GM1 staining was observed in Galgt1-/-mdx. The micron bar is 50µm.
Figure 3.24 Double immunostaining of 6 weeks old mdx and Galgt1-/mdx gastrocnemius with GM1 and Ertr7. GM1 does not co-stain with Ertr7 in mdx muscles and no GM1 staining was observed in Galgt1-/mdx. The micron bar is 50µm.

3.4. Discussion

The regenerative potential of an organ is an essential aspect by which it maintains its structure and function in the face of insults and injury. In genetic diseases that affect the skeletal muscle, such as the muscular dystrophies, where injury is chronic, robust muscle regeneration is essential to prevent muscle loss, or wasting, with its dire consequences to respiration, ambulation, and ultimately, life. In Duchenne muscular dystrophy (DMD), the regenerative capacity of the skeletal muscle is impaired (Watkins and Cullen, 1986) and ultimately is severely compromised, leading to increased disease
severity concomitant with loss of muscle tissue. Thus, the increased weakness and frailty of the sarcolemmal membrane in DMD is ultimately not offset by the ability to regenerate new muscle, which drives the progressive nature of the disease (Tedesco et al., 2010).

Satellite cells, the myogenic stem cell population of the muscle, are the key players in providing the muscle with the ability to regenerate and withstand injury, both during normal use and in disease, and a number of studies support an important role for gangliosides in their function. Eventually, due to a large number of cycles of division, the proliferative potential of the satellite cells decreases, both as the muscle ages and in DMD (Jejurikar and Kuzon, 2003; Schultz and Jaryszak, 1985). The actual number of satellite cells is also affected (Jejurikar and Kuzon, 2003; Schultz and Jaryszak, 1985).

Effective muscle regeneration can decrease the severity of muscular dystrophy tremendously. Galgt1 could be exploited therapeutically to alter regeneration. In absence of Galgt1, the regeneration of muscle is affected in both WT and mdx mice. There is a delay in muscle regeneration which leads to a delay in increase of myofiber diameter. We showed in this study that Galgt1 and GM1, a subsequent product of Galgt1, both are present in satellite cells in the days immediately after injury. This loss of Galgt1 expression alters regeneration kinetics. There are primarily four aspects of satellite cell biology that can be affected by loss of Galgt1: 1. It can affect satellite cell/myoblast proliferation, 2. It can affect satellite cell migration along the basal lamina,
3. It can affect differentiation of satellite cells into postmitotic myoblasts and 4, It can affect myoblast fusion into newly formed myotubes.

To assess the potential of complex gangliosides to control satellite cell growth, we overexpressed Galgt1 in C2C12 myotubes. In this experiment, we observed no difference in growth rate as compared to the non-transfected or GFP transfected controls (data not shown). Thus, it would appear that Galgt1 may not alter satellite cell growth, though studies in Galgt1-deficient cells are required here to prove this more directly. There have been studies in fibroblasts that indicate, although not conclusively proven, that increased ganglioside expression leads to inhibition of cell proliferation (Leskawa and Agranoff, 1983; Leskawa and Hogan, 1990). Many other studies suggest that gangliosides can alter intracellular signaling by kinases, which in theory could impact growth.

While there is no evidence that Galgt1 is required for satellite cell migration in regenerating muscle, there is ample evidence that glycans such as those found on complex gangliosides can mediate or modulate adhesion and migration of cells along extracellular matrix components. This work is discussed in detail in Chapters 1.

The processes of myoblast differentiation and myoblast fusion to create newly formed myotubes within the regenerating muscle are aspects of the regenerative process where Galgt1 may also have roles. For example, gangliosides present in lipid rafts can modulate myoblast membrane fluidity, which is important for membrane
fusion (Lee et al., 1980; Ollmann and Galla, 1985). GM1 is present on the lamellipodia of myoblasts (Mukai et al., 2009). The lamellipodia is where the membrane fusion takes place. The clustering and dispersal of lipid rafts is important for fusion competent myoblasts (Mukai et al., 2009). Many studies have indicated that glycoconjugates play an important role in myoblast recognition and fusion. Studies on fusion resistant fu-1 rat myoblasts cell line have shown that there is a consistent decrease in the levels of glycosphingolipids in these myoblasts and that they have also lost the ability to differentiate and fail to express the many markers of differentiating myoblast/myotubes (Cambron and Leskawa, 1994; Kaufman and Parks, 1977). Glycosylation inhibitors like tunicamycin (Gilfix and Sanwal, 1980), which inhibits glycosphingolipids, plant lectins (that binds specific carbohydrate structures) (Den et al., 1975) can also block myoblast fusion. Further, no fusion is observed in certain lectin-resistant mutants either (Gilfix et al., 1983; Parfett et al., 1983). All these studies indicate that glycosphingolipids and gangliosides in particular are involved with myoblast fusion and can possibly regulate differentiation of the myoblasts as well. Gangliosides are regulated differentially through the various stages of differentiation (Leskawa et al., 1988; Leskawa and Hogan, 1990). Since GD1a and GM1 are both increased in myoblasts just before fusion (Cambron and Leskawa, 1994), and as they both require Galgt1 for their synthesis, the increase in Galgt1 during regeneration can be important in myoblast fusion. During muscle regeneration, following acute muscle injury we observed that there were many more mononuclear cells present on day 7 and 14 after cardiotoxin injury in Galgt1-/-
when compared to WT, this apparent increase in mononuclear cells can be due to a
defect in myoblast fusion in absence of Galgt1 leading to an absence of all complex
gangliosides.

If expression of Galgt1 alters migration or fusion, it can have possible
implications in cell based therapy for Duchenne Muscular Dystrophy. In principle, these
therapies aim to restore functional dystrophin in the patient by injecting dystrophin
expressing transformed myoblasts into the patient muscle. In theory, the myoblasts will
then proliferate migrate and fuse to form new dystrophin expressing myofibers. This,
however, doesn’t happen with great efficiency using current technologies. Transplanted
donor myoblasts are rarely able to cross the basal lamina of the recipient muscle and do
not migrate to other parts of the muscle that are undergoing regeneration. These cells
also have extremely low incorporation into the existing myofibers and fuse poorly with
donor myoblasts. These shortcomings severely restrict the use of cell based therapies
for treatment of DMD (Bentzinger et al., 2010; Farini et al., 2009). But if Galgt1 affects
myoblast fusion and migration, it could be exploited to change the migratory and fusion
capacity of the cultured myoblasts. The levels of most gangliosides are reduced after
only a few days in culture, and this can hamper the ability of the myoblasts to fuse
efficiently or migrate across the basal lamina. It will be interesting to look at myoblasts
from DMD patients and see whether the loss of Galgt1 expression with continuous
cycles of division is decreased and whether this decrease in Galgt1 expression correlated
to fusion incompetent cells. Galgt1 can, therefore be a potential therapeutic target for
DMD. It can be co-transfected along with dystrophin in the myoblasts and can give the cell based therapy an edge. Alternatively, bioactive gangliosides could be directly co-delivered with various cell therapies to increase their migratory or differentiation properties.
Chapter 4. SUMMARY

Glycans are very diverse molecules with more combinations of sequences possible, per motor unit, than proteins or DNA. They can be added to lipids or proteins which then imparts important functional properties to those molecules. NMJ is a specialized region of the muscle membrane where many unique heavily glycosylated proteins and glycolipids are expressed. These glycans can mediate interactions between ECM and the pre- and post-synaptic membrane and mediate binding between ligand and receptors and ECM and secreted glycoproteins. The functions of specific glycan structures at the NMJ are very poorly understood.

In this thesis, we showed that the two glycosyltransferases that synthesize the same basic glycan structure on different substrates have distinct functional specificity. The products of Galgt1 and Galgt2 don’t show functional redundancy despite being similar in primary sequence. The overexpression of Galgt2 in mdx mice ameliorates muscular dystrophy and the CT glycan is expressed along the entire length of sarcolemma membrane. In contrast, Galgt1 overexpression doesn’t cause the expression of the sugars around the sarcolemma and in fact causes muscle pathology.
Galgt2 deletion leads to instability of AChRs at the NMJ whereas a Galgt1 deletion leads to a delay in muscle regeneration.

There is a natural overexpression of Galgt1 in satellite cell that occurs immediately after muscle injury. And the lack of Galgt1 from satellite cell causes a delay in muscle regeneration. We need to further elucidate the role of Galgt1 in muscle regeneration by studying what aspect of satellite cell biology is changed by the lack of Galgt1. To study this we will make myofiber cultures from WT and Galgt1-/- mice and will study the satellite cell proliferation, differentiation, migration and fusion in myofiber culture with respect to Galgt1 and GM1 expression in WT satellite cells. This will help us understand the exact role of Galgt1 in satellite cell/ myoblast biology and can be exploited to design better myogenic precursor cells for cell based therapies.

It will also be interesting to study, why overexpression of Galgt1 in myofibers leads to muscle pathology and decrease in myofiber diameter. There is some indication that gangliosides can lead to inhibition of cell proliferation. It is possible that this may explain why myofiber size is decreased when Galgt1 was postnatally overexpressed. It is also possible that overexpression of Galgt1 in myofibers leads to an accumulation of gangliosides which leads to toxicity. Ganglioside accumulation in motor neurons causes severe neurological disorders like Tay Sach’s. This can be investigated by staining for various simple gangliosides upstream of Galgt1.
Determination of the role of Galgt2 in stabilization of AChRs at the NMJ will add significantly to our understanding of glycan biology at NMJ and to the mechanism by which Galgt2 works. Galgt2 glycosylates dystroglycan at the NMJ and dystroglycan stabilizes the AChR receptors at the NMJ. The internalization of AChRs in absence of Galgt2 could be due to the fact the lack of CT antigen on dystroglycan changes its binding properties at the neuromuscular junction. As such, Galgt2 overexpression at the synapse may be a means by which the normal degeneration of neuromuscular morphology that occurs in the aging muscle can be overcome or treated.
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124
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