Published in final edited form as:

ACS Chem Neurosci. 2018 December 19; 9(12): 3086–3093. doi:10.1021/acscchemneuro.8b00295.

Influencing Early Stages of Neuromuscular Junction Formation through Glycocalyx Engineering

Mia L. Huang[†], Ember M. Tota, Taryn M. Lucas, and Kamil Godula^{*}

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0358, United States

Abstract

Achieving molecular control over the formation of synaptic contacts in the nervous system can provide important insights into their regulation and can offer means for creating well-defined in vitro systems to evaluate modes of therapeutic intervention. Agrin-induced clustering of acetylcholine receptors (AChRs) at postsynaptic sites is a hallmark of the formation of the neuromuscular junction, a synapse between motoneurons and muscle cells. In addition to the cognate agrin receptor LRP4 (low-density lipoprotein receptor related protein-4), muscle cell heparan sulfate (HS) glycosaminoglycans (GAGs) have also been proposed to contribute to AChR clustering by acting as agrin co-receptors. Here, we provide direct evidence for the role of HS GAGs in agrin recruitment to the surface of myotubes, as well as their functional contributions toward AChR clustering. We also demonstrate that engineering of the myotube glycocalyx using synthetic HS GAG polymers can replace native HS structures to gain control over agrin-mediated AChR clustering.

Graphical Abstract

Keywords

neuromuscular junction; AChR clustering; agrin; heparan sulfate; glycocalyx engineering

^{*}Corresponding Author, kgodula@ucsd.edu.

[†]Present Address, 130 Scripps Way, Jupiter, Florida 33458-5284, United States

Author Contributions

M.L.H. and K. G. designed research; M.L.H., E.M.T., and T.M.L. performed experiments. M.L.H., E.M.T., T.M.L., and K.G. analyzed data, M.L.H., E.M.T., and K.G. wrote the paper.

Notes

The authors declare no competing financial interest.

INTRODUCTION

The neuromuscular junction (NMJ) is a chemical synapse formed between a motor nerve and a muscle fiber. Proper NMJ development ensures that action potentials are transmitted from the nervous to the muscular system with high fidelity, allowing for precise control of muscle movement. The formation of the NMJ is a carefully orchestrated multistep process, which begins during early development with the innervation of muscle fibers and the formation of synaptic connections and continues over several weeks postnatally.^{1,2} Dysregulation of the synaptogenic process results in diseases, such as congenital myasthenia and myasthenia gravis,^{3,4} manifested by impaired contractility and wasting of muscles. In adults, deterioration of the synapse accompanies auto-immune disorders, such as Isaac's and Lambert—Eaton syndromes,⁵ and occurs naturally due to aging.^{6,7} Therefore, in vitro models of both healthy and diseased states of the NMJ have been sought to aid in identifying and evaluating possible modes of pharmacological intervention to address the various afflictions of the synapse.

Early work using in vitro coculture models,^{8,9} recently advanced to include human stem cell-derived systems,^{10–14} which aim to recapitulate the characteristics of native NMJs, has pointed to the importance of proper organization of the synapse to achieve efficient signal transduction from motoneurons and regularity of muscle contraction. Engineering strategies have been developed to improve the performance of in vitro NMJ models,^{15–17} primarily using microfabrication techniques for the alignment of muscle fibers and precise positioning of muscle—motoneuron contacts^{18,19} or by fine-tuning the mechanical properties of the culture matrix.²⁰ Chemical methods for manipulating the early signaling events that lead to the organization of the synapse may provide molecular-level tools for constructing in vitro NMJ models that complement and enhance existing engineering approaches.

During the innervation of muscle tissues, motor nerve terminals secrete a macromolecular proteoglycan, agrin,²¹ which interacts with the low density lipoprotein receptor-related protein 4 (LRP4) associated in a complex with muscle-specific tyrosine kinase (MuSK, Figure 1).^{22–25} Activation of the LRP4/MuSK complex, in turn, enhances the accumulation and stabilization of acetylcholine receptor (AChR) clusters in the postsynaptic membrane.^{26–29} Agrin, which is essential in this process, is highly regulated by mRNA splicing, and its variants can be found across several tissues, including the immune and muscular systems.^{30–32} Only the nerve derived splice variant, referred to as the A/ γ ⁺ and B/ z ⁺ agrin, is able to cluster AChRs.^{33–36} The B/ z site encodes for an amino acid sequence with 8–19 residues that interacts with the muscle receptor LRP4. The A/ γ site contains a shorter, positively charged tetrapeptide, KSRK, that has been identified as a binding site for heparin, a highly sulfated member of the heparan sulfate (HS) family of glycosaminoglycans (GAGs).^{37–39}

Ample evidence points to the contributions of HS on the surface of myofibers to agrin-mediated AChR clustering.^{40–44} For instance, soluble heparin, HS, and other polyanions that can disrupt HS—protein interactions have been shown to inhibit agrin induced AChR clustering.⁴² Heparin has also been shown to block agrin-dependent AChR clustering and functional innervation in muscle—neuron cocultures.⁴⁵ Further evidence for the functional role of HS GAGs is provided by studies reporting diminished agrin-mediated AChR

clustering on muscle cells with reduced levels of GAG sulfation, either caused by mutations in proteoglycan biosynthesis^{36,43} or induced chemically by treatment with chlorate anions,⁴⁴ a general inhibitor of cellular sulfation. Collectively, these studies provide evidence that the glycocalyx of muscle cells harbors binding sites for agrin, which may be exploited to manipulate the process of AChR aggregation in cultured myotubes and, possibly, control nerve-induced NMJ formation in vitro. We have established and report here a conceptually simple process for controlling AChR clustering in myotubes by removing intrinsic binding sites for agrin and remodeling their glycocalyx with synthetic agrin co-receptors based on well-defined nanoscale glycomaterials designed to recapitulate the architectural and functional features of native HS.

RESULTS

The Myotube Glycocalyx Harbors HS GAG Co-receptors for Agrin.

During the nerve-induced phase of NMJ formation, agrin secreted by motoneurons is required to increase recruitment of AChRs and their organization into dense clusters in the postsynaptic terminal regions on myofibers (Figure 1). Whereas LRP4 has been identified as the primary myotube receptor for agrin,^{23,24} the addition of polyanions can inhibit the process of AChR clustering. Similar effects have been observed in myotubes exhibiting defects in HS GAG structures due to chemical and genetic perturbations of their biosynthetic assembly. These findings suggest that HS GAGs may be directly involved in agrin binding and can serve as a point of chemical intervention to manipulate the AChR clustering process and, thus, to acquire control over the formative step in NMJ development.

To provide direct evidence for agrin binding to HS GAGs in the myotube glycocalyx, we have evaluated the binding of recombinant C-terminal agrin containing the *A/y* splice insert required for HS binding^{37–39} to differentiated C2C12 mouse skeletal muscle myotubes (Figures S1 and S2). C2C12 myoblasts were differentiated into myotubes over 4–6 days,^{46,47} during which the mononucleated myoblasts fused into long multinucleated myotubes (Figure S3). Using a cellular ELISA, we observed binding of agrin to the myotube surface, which reached saturation at 200 nM agrin (Figure 2A). To determine the contributions of HS GAG structures at the cell surface to agrin binding, we first added heparin and HS as soluble competitors (Figure 2B). Heparin (Figure S4) strongly inhibited the agrin—myotube interaction in a dose-dependent manner, with ~25% loss of binding at 0.2 $\mu\text{g/mL}$ and ~50% loss of binding at 50 $\mu\text{g/mL}$. Further enhancements of inhibition were not observed with higher concentrations of heparin. The less sulfated HS provided only ~25% inhibition of agrin binding at a 50 $\mu\text{g/mL}$ concentration. The inability of heparin and HS to fully inhibit agrin binding is consistent with the presence of other high-affinity agrin receptors on the myotube surface, such as LRP4.²³ To further confirm the contributions from cell surface HS to agrin binding, we selectively removed endogenous HS structures from the myotube glycocalyx with heparinase enzymes (Figure 2C). Treatment of myotubes with a cocktail of heparinase (I–III) enzymes (1.7 mIU) led to a significant loss of cell surface HS mass (Figure S5A) and HS immunofluorescence (Figure 2C and Figure S5B). This loss was accompanied by a significant decrease in agrin association with the cell surface compared to untreated cells, as observed by both immunostaining and cellular ELISA (Figure 2C,D and

Figure S5C). As expected, neither the inclusion of soluble competitors nor heparinase treatment were able to completely abolish agrin binding to myotubes, consistent with the lack of overlap between the putative HS and LRP4 receptor binding domains of agrin.^{23,38}

Elimination of HS Co-receptors of Agrin from Myotube Glycocalyx Impairs AChR Clustering.

To investigate the functional consequences of the loss of cell surface HS on the organization of postsynaptic terminals of the NMJ, we performed in vitro assays evaluating the recruitment and organization of AChRs in response to agrin. Addition of exogenous agrin (5 nM) for 18 h to differentiated myotubes in culture led to the formation of large (~5 μm) clusters of AChRs, which were visualized by staining with a AF488-labeled α-bungarotoxin (Figure 2E). Low levels of AChR clustering were observed without the addition of agrin due to the ability of myotubes to spontaneously cluster AChRs.⁴⁸ To provide a semiquantitative assessment of agrin-induced AChR clustering, we collected multiple (~10) fluorescence images per condition and manually counted punctate fluorescent spots of AChR clusters of ~5 μm size or larger (Figure S6). Next, we assessed the formation of agrin-induced AChR clusters in myotubes with enzymatically altered surface HS GAG structures and in the presence of soluble GAG inhibitors of agrin binding (Figure 2F). Treatment of myotubes with heparinases (I–III) prior to agrin addition significantly attenuated the number of AChR clusters formed (Figures 2F and S7). We observed some residual AChR clustering activity, which may stem from HS-independent binding of agrin to LRP4 (Figure 2D) or, alternatively, from partial recovery of HS GAG expression over the duration of the AChR clustering assay. The addition of soluble heparin (0.05–50 μg/mL) also significantly impaired AChR clustering (Figure 2F). These observations provide evidence for the contributions of myotube surface HS structures toward the formation of AChR clusters in response to agrin stimulation.

Agrin Exhibits High-Affinity Binding to Moderately Charged HS Oligosaccharides with Appropriate Combinations of *N*-, 2-*O*-, and 6-*O*-Sulfation.

We next sought to determine the structural determinants within natural HS GAGs necessary for high-affinity agrin binding. The protein-binding regions of HS GAG polysaccharides are composed of alternating disaccharides repeats comprising iduronic or glucuronic acid residues linked to glucosamine, which can be sulfated at the C2 hydroxyl of the uronic acid or the C6 and C3 hydroxyl or the amino groups of glucosamine (Figure 1). The negatively charged sulfate groups are recognized by proteins, such as agrin, and the unique spatial organization of charges along the HS GAG chains gives rise to specificity in protein recognition.⁴⁹ To identify sulfation patterns of HS GAGs required for agrin binding, we utilized a microarray of 24 synthetic HS structures that differed in length and sulfation (Figure 3). Included in this library were HS structures spanning from tetrasaccharides to nonasaccharides free of sulfate groups or containing combinations of *N*-sulfate, 6-*O*-sulfate, 2-*O*-sulfate, or 3-*O*-sulfate modifications (Figure 3 and Figure S8). Analysis of agrin (50 nM) binding against this array revealed that moderate levels of sulfation in HS are required for high-affinity interactions. The nonsulfated analogs (HS1–6) exhibited little agrin binding regardless of chain length, which was marginally improved upon the introduction of *N*-sulfates (HS7–12). The inclusion of up to four additional 6-*O*-sulfates (HS13–16) or one 2-

O-sulfate (HS17) continued to improve agrin association. The highest affinity interaction was observed between agrin and a fully *N*-sulfated HS octasaccharide carrying four 6-*O*-sulfate groups and a single 2-*O*-sulfation (HS20). Interestingly, the introduction of more than one 2-*O*-sulfate or additional 3-*O*-sulfation (HS21–22 and HS23–24, respectively) caused decreases in binding, thus revealing the preference of agrin for moderately charged *N*-sulfated HS oligosaccharides with additional appropriate combinations of 2-*O*- and 6-*O*-sulfation patterns.

Our cell-surface and glycan array binding data, together with AChR clustering assays, provide direct evidence for the functional role of HS GAGs as co-receptors for agrin in the myotube glycocalyx, where they work in concert with the LRP4 receptor to promote AChR recruitment and synapse organization. As such, myotube HS GAGs provide opportune targets for chemical manipulation of the neuromuscular synaptogenic process.

Synthetic HS-Mimetic Materials Engage Agrin.

We next asked whether the engineering of myotubes with synthetic agrin co-receptors could recapitulate the function of endogenous HS GAG structures. In their work, Hsieh-Wilson and co-workers showed that sulfated GAG disaccharides can recapitulate the binding profiles and biological activities of their parent GAGs when arranged in multivalent ensembles on linear polymer scaffolds.^{50,51} For instance, glycopolymers carrying the disaccharide motif of chondroitin sulfate E (UA-GalNAc4,6S) were able to inhibit hippocampal neuron outgrowth in culture at levels comparable to the native polysaccharide.⁵¹ We have developed a rapid and modular synthesis of GAG-mimetic glycopolymers using direct conjugation of reducing GAG disaccharides to polymer scaffolds decorated with side chains containing reactive α -heteroatom nucleophiles, such as the *N*-methylaminoxy group.⁵² This method produced a set of glycopolymers representing the sulfation patterns found in native HS structures using sulfated GAG disaccharides generated by lyase depolymerization of HS polysaccharides.⁵² Inclusion of these materials into glycan array platforms enabled rapid analysis of their interactions with HS-binding proteins. Endowing the polymers with a phospholipid anchoring element⁵³ permitted the delivery of these materials directly into the glycocalyx of mouse embryonic stem cells, where they promoted fibroblast growth factor signaling and neural differentiation.⁵²

Armed with the structure–activity relationship information obtained from the HS array (Figure 3) that pinpointed a preference of agrin to bind moderately sulfated HS regions containing *N*-, 2-*O*-, and 6-*O*-sulfation, we decided to generate azido-functionalized synthetic HS mimetics 1 (Scheme S1) with ~150–200 repeating units (*n*) and low chain length distributions ($\text{MWD} = 1.15$). We chose to generate polymers presenting either the 2-*O*- and 6-*O*-disulfated HS disaccharide D2A6⁵⁴ (UA2S-GlcNAc6S, 1a), as a possible agrin-binding motif, or the monosaccharide *N*-acetylglucosamine-6-*O*-sulfate (GlcNAc6S, 1b), which we anticipated to have no appreciable affinity toward agrin despite its highly anionic character (Figure 4A). Based on our HS glycan array data (Figure 3), the *N*-, 2-*O*- and 6-*O*-trisulfated HS disaccharide D2S6 (UA2S-GlcNS,6S) may appear to present a more obvious choice as an agrin recognition element in our mimetic. However, in our prior work, we observed that binding of growth factors (e.g., FGF2) to polymers carrying D2A6

disaccharides was more effective due to their increased glycan ligation efficiency and higher resistance to aggregation in solution, compared to polymers carrying D2S6 glycans.⁵²

Both D2A6 and GlcNAc6S glycans were efficiently ligated (74% and 83%, respectively) to the reactive azido polymer backbone containing *N*-methylaminooxy side chains and a fluorescent (AlexaFluor488) tag under mildly acidic conditions (Table S1 and Figure S9). We then immobilized both mimetics onto cyclooctyne-functionalized glass slides (Scheme S2) using strain-promoted copper-free click chemistry⁵⁵ to evaluate their ability to engage soluble agrin. Only glycopolymer 1a presenting the D2A6 disaccharide generated a robust agrin binding response. We observed no appreciable agrin binding to the control glycopolymer 2b (Figure S10). Quantitative analysis of glycopolymer binding to agrin was obtained using surface plasmon resonance (SPR) with biotinylated versions of D2A6 (2a) and GlcNAc6S (2b) glycans (Table S1, Figure 4B). Glycopolymer 2a bound to surface-immobilized agrin with an apparent dissociation constant ($K_{D,app}$) of 6.0 nM similar to that for heparin ($K_{D,app}$ = 1.6 nM, Figure S11). No measurable avidity was observed for the control glycopolymer 1b even at high (900 nM) concentrations (Figure 4B). Thus, with the inclusion of the D2A6 glycan into the polymer scaffold, we were able to approximate the agrin-binding capability of the native heparin polysaccharide.

HS GAG-Mimetic Polymers Act As Functional Synthetic Co-receptors for Agrin in the Myotube Glycocalyx.

The HS mimetics were primed for insertion into the outer leaflet of myotube plasma membranes by inclusion of a phospholipid tail to one end of the polymer chain (glycopolymers 3a and 3b, Figure 4A) The glycocalyx of differentiated myotubes was remodeled in two steps consisting of the overnight removal of endogenous HS structures with a heparinase (I–III) cocktail, followed by incubation with lipidated glycopolymers 3a or 3b (5 μ M, Figure 4C) for 1 h. After membrane incorporation, the cells were washed to remove unbound polymers. Analysis of the remodeled cells by fluorescence microscopy indicated robust incorporation of both mimetics to the cellular plasma membrane (Figure S12). Microscopic evaluation revealed minimal to no deleterious effects of the remodeling process on cell morphology or viability. To assess the functional consequences of the glycocalyx remodeling process, the myotubes were subjected to an AChR clustering assay in the presence of agrin, as described above (Figure 4D; Figure S13). Only the **D2A6 HS-mimetic 3a** was able to rescue **AChR** clustering compared to cells only treated with heparinase at identical time points. This observation is consistent with the agrin-binding profiles of the polymers and demonstrates the ability of the glycopolymer **3a** to act as a functional surrogate of native HS GAG polysaccharides on the myotube surface enabling chemical manipulation of the AChR clustering process. The inhibition of AChR clustering in the heparinase treated control and its rescue after the introduction of 3a appear to also point to the requirement for early onset of agrin activity in the AChR clustering process, since both recovery of endogenous cell-surface HS and clearance of glycopolymers 3b are expected over the duration of the experiment. Levels of AChR clustering did not improve after treatment with 3b compared to heparinase treated cells, indicating a lack of ability of this polymer to facilitate agrin activity at the myotube surface.

DISCUSSION

Glycans, such as HS GAGs, have been increasingly identified as mediators of signaling at the cell surface, where they serve as co-receptors for a variety of extracellular biochemical cues. Synthetic glycomaterials have provided essential tools for investigating and manipulating signal transduction via glycans at the cellular boundary,⁵⁶ and the integration of glycomaterials with cell surface engineering approaches⁵⁷ may open new opportunities for tailoring glycan interactions within the cellular glycocalyx to orchestrate complex morphological events, such as the formation and organization of the neuromuscular junction (NMJ). In the most recent model of NMJ development,² binding of agrin to its receptor, LRP4, is required to initiate signaling events leading to the organization of the postsynaptic apparatus. Motoneurons secrete agrin, and its local deposition results in transduction of the signal from LRP4 to the associated tyrosine kinase, MuSK, and activation of a downstream signaling cascade that ultimately results in the accumulation of localized AChRs in the postsynaptic membrane. It is apparent that agrin activity is highly regulated by mRNA splicing and that neural agrin containing both the *A/y* and the *B/z* splice sites (Figure 1) is the most potent in inducing AChR clustering.^{34,58} The *B/z* site is largely responsible for binding to LRP4,²⁵ whereas the *A/y* site has been determined to present a binding site for HS GAG polysaccharides.^{37–39} Agrin isoforms, including muscle agrin, that lack a functional *A/y* site have been shown to have reduced AChR clustering.³⁶ Our results corroborate previous studies using affinity-based analytical techniques that established that the *A/y*⁺ splice variant of agrin, bearing the KSRK amino acid sequence, bound to these polysaccharides.^{37–39} In this study, we strengthen these relationships toward the HS members of the GAG family, by demonstrating their contributions to enhancing agrin binding at the surface of differentiated murine muscle myotubes, where the main receptor of agrin, LRP4, is also present.

Enhanced association of agrin with the myotube surface is expected due to the presence of LRP4, as well as putative (HS GAG or α -dystroglycan)^{59,60} and, possibly, other yet unidentified receptors. Our cell-based ELISA bore out this expectation, showing a dose-dependent agrin binding to the myotube surface (Figure 2A). To isolate the contributions of HS GAG co-receptors to agrin binding, we introduced soluble competitors (heparin or HS) for the myotube surface GAG structures (Figure 2B) and observed a significant inhibition of agrin binding to the myotubes in the presence of both polysaccharides. The more anionic heparin molecule was a stronger inhibitor of agrin-binding to the cell surface compared to the less sulfated HS, possibly pointing to the importance of ionic interactions in agrin association; however, our HS glycan array binding data do not support this limited conclusion. While the set of arrayed oligosaccharides may be limited with respect to the expected molecular diversity of endogenous HS structures, it nonetheless revealed a preference of agrin for engaging structures containing combinations of *N*-, 2-*O*- and 6-*O*-sulfation (Figure 3). Whereas increasing 6-*O*-sulfation generally enhanced agrin binding, the presence of more than one 2-*O*-sulfate or a 3-*O*-sulfate, which are characteristic features of heparin, negatively impacted agrin association, indicating a requirement for a moderate density and optimal presentation of sulfate groups for high avidity binding of agrin. Therefore, the enhanced ability of heparin to inhibit agrin binding to cell surface HS more

likely arises from a higher effective number of binding sites for agrin on the soluble heparin polysaccharide chain compared to HS, which typically contains fewer highly sulfated domains flanked by regions of low or no sulfation.

The removal of endogenous HS structures from the myotube glycocalyx by treatment with heparinase enzymes also significantly decreased agrin binding. While agrin binding was not fully eliminated, which we ascribe to the non-overlapping LRP4 and HS binding regions in agrin,^{37–39} our observations point to the relevance of HS interactions in mediating agrin recruitment to the cell surface (Figure 2B–D). These treatments either remove or compete for sulfated HS GAG structures in the cellular glycocalyx and their observed negative effects on the ability of agrin to induce clustering of AChRs in differentiated myotube culture (Figure 2E,F) mirror prior observations by McDonnell et al.⁴⁴ HS proteoglycans (HSPGs) are well-known constituents of the myotube glycocalyx,⁶¹ and especially, HS chains with enhanced 2-*O*- and 6-*O*-sulfation are a prominent feature at the muscle cell surface.^{41,62} Although specific myotube HSPG receptors that interact with agrin, and possibly also its receptor LRP4, to influence AChR clustering are yet to be identified, the HSPG perlecan has been shown to colocalize with AChR clusters, and the addition of agrin significantly enhanced the amount of perlecan colocalization.⁶³ Based on their contributions to the early phases of NMJ development, HS GAGs provide a unique and accessible target for chemical manipulation of this process. We were able to reconstitute agrin-binding activity in synthetic glycopolymers bearing a 2-*O*- and 6-*O*-disulfated HS disaccharide, D2A6, (Figure 4B) and deliver these materials to the surfaces of myotubes devoid of functional HS GAGs by passive membrane insertion via a lipid anchor (Figure 4D). The opposing effects of soluble heparin and HS polysaccharides, which inhibit agrin-mediated AChR clustering (Figure 2B), and the cell surface-presented glycopolymers that promote cluster formation (Figure 4D) highlight the importance of proper localization of the glycomimetic activity within the cellular glycocalyx.⁶⁴

While the D2A6 disaccharide served as an effective agrin binding element in our polymers, the ability to modify the polymer scaffolds with more complex chemically defined HS oligosaccharides containing native-like constellations of sulfate patterns, which are becoming broadly available with recent advances in chemoenzymatic glycosaminoglycan synthesis,⁶⁵ may provide further opportunities for fine-tuning of agrin activity. Our microarray data indicates a requirement for an optimal level of sulfation for agrin binding, suggesting that exploring synthetic polymers presenting GAG structures beyond the limited set investigated in this work may yield materials with enhanced ability to promote or even inhibit agrin-dependent AChR clustering. Selective glycopolymer inhibitors of agrin activity would constitute a particularly valuable tool for controlling NMJ formation in more complex multicellular environments, such as motoneuron cocultures or engineered tissue scaffolds, where the global removal of background cell surface HS activity with heparinase treatment may influence other cellular functions.

The current study focuses on the interactions of cell-surface HS with agrin; however, it should be noted that other protein components of the agrin—receptor complex may also harbor HS-binding domains sensitive to changes in sulfation, further extending the role of the glycocalyx in regulating AChR organization and patterning. The ability to manipulate

this key early step in the formation of the NMJ synapse through facile alteration of the myotube glycocalyx composition provides a molecular-level tool that complements and may enhance current engineering strategies being applied toward developing functional NMJ models and enable further study of the physiology and disease of the synapse.

METHODS

Tissue Culture.

The C2C12 (ECACC no. 91031101, procured through Sigma-Aldrich no. 91031101) mouse skeletal muscle cell line was used for all experiments. Cells were propagated as undifferentiated myoblasts in DMEM + 10% FBS growth medium. For experiments, myoblasts were predifferentiated into myotubes for 4–6 days in DMEM + 2% horse serum.

Synthesis of HS Mimetics.

The HS mimetic glycopolymers were synthesized as previously described (Scheme S1).⁵² Briefly, an *N*-methylaminoxyacrylamide monomer was polymerized with an azide-, phospholipid- or a biotin-chain transfer agent via the RAFT method to generate low dispersity (≈ 1.15) polymer backbones. Following chain end trithiocarbonate deprotection, fluorophore labeling, and side-chain Boc-group deprotection, HS disaccharides were conjugated to the reactive *N*-methylaminoxy side chains under acidic conditions (pH = 4.5) for 3 days at 50 °C, and subsequent purification yielded HS mimetic glycopolymers.

Cell-Based Enzyme-Linked Immunosorbent Assay (ELISA) To Determine Agrin Binding.

For agrin competition assays with soluble HS GAG polysaccharides, 10 nM agrin was added to titrations of heparin or HS and premixed prior to addition to myotubes. For conditions requiring removal of surface HS structures, myotubes were treated overnight with heparinase I, II, and III (1.7 mIU per well) prior to probing with agrin (10 nM) as described above. HS levels and the extent of agrin binding were determined based on the enzymatic activity of the HRP-linked secondary antibodies bound to the anti-HS or anti-agrin primary antibodies, respectively.

Microarray Printing and Analysis.

Epoxy-functionalized micro-array glass slides were prefunctionalized with cyclooctyne-amine prior to printing (Scheme S2). Solutions containing HS-mimetic glycopolymers 1a and 1b bearing azide groups were arrayed on the functionalized substrates using a robotic ArrayIt spotter. After overnight reaction and washing, the glass slides were incubated with solutions of agrin, and binding was probed with a primary anti-agrin antibody, followed by a fluorophore-labeled secondary antibody. Fluorescence intensities were measured with a Genepix 4000B array scanner.

SPR Analysis.

Agrin (300 nM) was covalently immobilized on carboxylic acid-functionalized gold nanoparticle SPR chips. After blocking, biotinylated HS mimetics were flowed over the immobilized agrin in running buffer and allowed to dissociate. A blank injection of running

buffer was performed and subtracted from the signal. The surface was regenerated between HS mimetic injections.

AChR Clustering Assays.

Mononucleated myoblasts seeded in 24-well plates were allowed to differentiate for 4–6 days to form robust multinucleated myotubes. For experiments requiring the removal of surface HS structures, myotubes were treated with heparinase I, II, and III (1.7 mIU per well) overnight in DMEM prior to subsequent treatments. For cells remodeled with glycopolymers, heparinase-treated myotubes were preincubated for 1 h with the glycopolymers diluted in DMEM, and extensively washed with PBS. The myotubes were then treated with recombinant C-terminal agrin overnight (~18 h, 5 nM) at 37 °C. In our experiments, continued heparinase treatment during glycopolymer incubation led to similar outcomes as when the enzyme is not present. After washing with PBS, cells were treated live with TAMRA-labeled α -bungarotoxin (1 h at 37 °C). Cells were then fixed and imaged with a fluorescence microscope. Ten images per well ($N = 10$) were collected at 20 $\times$ magnification, and the number of AChR clusters were manually counted per image. Only AChR clusters greater than 5 μ m were used for analysis. Bar graphs represent the mean $\pm$ SEM per condition. Experiments were conducted with at least three biological replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We are grateful to Christopher Fisher and Dr. Yinan Wang for assistance with polymer synthesis. We also acknowledge Biswa Choudhary of the GlycoAnalytics Core Facility of the UC San Diego Glycobiology Research and Training Center for analysis of GAG preparations.

Funding

K.G. is supported by the Alfred P. Sloan Foundation and the Research Corporation for Science Advancement. This work was supported, in part, by the NIH Director's New Innovator Award (NICHD, 1DP2HD087954-01). M.L.H. is supported by the NIH Pathway to Independence Award (NICHD, 1K99HD090292-01). E.M.T. is supported by the National Science Foundation Graduate Research Fellowship Program. T.M.L. is supported by Chemical Biology Interfaces Program (NIGMS, 5T32GM112584-03). M.L.H. and K.G. are supported in part by the Program of Excellence in Glycosciences (PEG, NHLBI, 5P01HL107150-07).

REFERENCES

- (1). Shi L, Fu AK, and Ip NY (2012) Molecular mechanisms underlying maturation and maintenance of the vertebrate neuro-muscular junction. *Trends Neurosci* 35 (7), 441–453. [PubMed: 22633140]
- (2). Witzemann V (2006) Development of the neuromuscular junction. *Cell Tissue Res* 326 (2), 263–271. [PubMed: 16819627]
- (3). Engel AG, and Sine SM (2005) Current understanding of congenital myasthenic syndromes. *Curr. Opin. Pharmacol* 5 (3), 308–321. [PubMed: 15907919]
- (4). Beeson D, et al. (2008) Congenital myasthenic syndromes and the formation of the neuromuscular junction. *Ann. N. Y. Acad. Sci* 1132, 99–103. [PubMed: 18567858]
- (5). Hirsch NP (2007) Neuromuscular junction in health and disease. *Br. J. Anaesth* 99 (1), 132–138. [PubMed: 17573397]

- (6). Li Y, Lee Y il, and Thompson WJ (2011) Changes in aging mouse neuromuscular junctions are explained by degeneration and regeneration of muscle fiber segments at the synapse. *J. Neurosci* 31 (42), 14910–14919. [PubMed: 22016524]
- (7). Tintignac LA, Brenner H-R, and Rüegg MA (2015) Mechanisms Regulating Neuromuscular Junction Development and Function and Causes of Muscle Wasting. *Physiol. Rev* 95 (3), 809–852. [PubMed: 26109340]
- (8). Das M, et al. (2007) Embryonic motoneuron-skeletal muscle co-culture in a defined system. *Neuroscience* 146 (2), 481–488. [PubMed: 17383103]
- (9). Das M, Rumsey JW, Bhargava N, Stancescu M, and Hickman JJ (2010) A defined long-term in vitro tissue engineered model of neuromuscular junctions. *Biomaterials* 31 (18), 4880–4888. [PubMed: 20346499]
- (10). Guo X, Gonzalez M, Stancescu M, Vandenberg HH, and Hickman JJ (2011) Neuromuscular junction formation between human stem cell-derived motoneurons and human skeletal muscle in a defined system. *Biomaterials* 32 (36), 9602–9611. [PubMed: 21944471]
- (11). Umbach JA, Adams KL, Gundersen CB, and Novitch BG (2012) Functional neuromuscular junctions formed by embryonic stem cell-derived motor neurons. *PLoS One* 7 (5), e36049. [PubMed: 22574134]
- (12). Chipman PH, Zhang Y, and Rafuse VF (2014) A stem-cell based bioassay to critically assess the pathology of dysfunctional neuromuscular junctions. *PLoS One* 9 (3), e91643. [PubMed: 24626225]
- (13). Steinbeck JA, et al. (2016) Functional Connectivity under Optogenetic Control Allows Modeling of Human Neuromuscular Disease. *Cell Stem Cell* 18 (1), 134–143. [PubMed: 26549107]
- (14). Demestre M, et al. (2015) Formation and characterisation of neuromuscular junctions between hiPSC derived motoneurons and myotubes. *Stem Cell Res* 15 (2), 328–336. [PubMed: 26255853]
- (15). Ko IK, et al. (2013) The effect of in vitro formation of acetylcholine receptor (AChR) clusters in engineered muscle fibers on subsequent innervation of constructs in vivo. *Biomaterials* 34 (13), 3246–3255. [PubMed: 23391495]
- (16). Tong Z, et al. (2014) Engineering a functional neuro-muscular junction model in a chip. *RSC Adv* 4 (97), 54788–54797.
- (17). Morimoto Y, Kato-Negishi M, Onoe H, and Takeuchi S (2013) Three-dimensional neuron-muscle constructs with neuro-muscular junctions. *Biomaterials* 34 (37), 9413–9419. [PubMed: 24041425]
- (18). Uzel SG, et al. (2016) Microfluidic device for the formation of optically excitable, three-dimensional, compartmentalized motor units. *Sci. Adv* 2 (8), e1501429. [PubMed: 27493991]
- (19). Ostrovidov S, et al. (2017) Three-dimensional co-culture of C2C12/PC12 cells improves skeletal muscle tissue formation and function. *J. Tissue Eng. Regener. Med* 11 (2), 582–595.
- (20). Happe CL, Tenerelli KP, Gromova AK, Kolb F, and Engler AJ (2017) Mechanically patterned neuromuscular junctions-in-a-dish have improved functional maturation. *Mol. Biol. Cell* 28 (14), 1950–1958. [PubMed: 28495800]
- (21). Christian CN, et al. (1978) A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells. *Proc. Natl. Acad. Sci. U. S. A* 75 (8), 4011–4015. [PubMed: 279017]
- (22). Weatherbee SD, Anderson KV, and Niswander LA (2006) LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. *Development* 133 (24), 4993–5000. [PubMed: 17119023]
- (23). Kim N, et al. (2008) Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell* 135 (2), 334–342. [PubMed: 18848351]
- (24). Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, and Mei L (2008) LRP4 serves as a coreceptor of agrin. *Neuron* 60 (2), 285– 97. [PubMed: 18957220]
- (25). Zong Y, et al. (2012) Structural basis of agrin-LRP4-MuSK signaling. *Genes Dev* 26 (3), 247–258. [PubMed: 22302937]
- (26). Tezuka T, et al. (2014) The MuSK activator agrin has a separate role essential for postnatal maintenance of neuromuscular synapses. *Proc. Natl. Acad. Sci. U. S. A* 111 (46), 16556–16561. [PubMed: 25368159]

- (27). Glass DJ, et al. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation and is a functional receptor for agrin. *Cold Spring Harbor Symp. Quant. Biol* 61, 435–444. [PubMed: 9246472]
- (28). Glass DJ, et al. (1996) Agrin acts via a MuSK receptor complex. *Cell* 85 (4), 513–523. [PubMed: 8653787]
- (29). Valenzuela DM, et al. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* 15 (3), 573–584. [PubMed: 7546737]
- (30). Ruegg MA, et al. (1992) The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. *Neuron* 8 (4), 691–699. [PubMed: 1314621]
- (31). Hoch W, Ferns M, Campanelli JT, Hall ZW, and Scheller RH (1993) Developmental regulation of highly active alternatively spliced forms of agrin. *Neuron* 11 (3), 479–490. [PubMed: 8398141]
- (32). Daniels MP (2012) The role of agrin in synaptic development, plasticity and signaling in the central nervous system. *Neurochem. Int* 61 (6), 848–853. [PubMed: 22414531]
- (33). Hoch W, Campanelli JT, Harrison S, and Scheller RH (1994) Structural domains of agrin required for clustering of nicotinic acetylcholine receptors. *EMBO J* 13 (12), 2814–2821. [PubMed: 8026466]
- (34). Gesemann M, Denzer AJ, and Ruegg MA (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *J. Cell Biol* 128 (4), 625–636. [PubMed: 7860635]
- (35). Bezakova G, and Ruegg MA (2003) New insights into the roles of agrin. *Nat. Rev. Mol. Cell Biol* 4 (4), 295–308. [PubMed: 12671652]
- (36). Ferns MJ, Campanelli JT, Hoch W, Scheller RH, and Hall Z (1993) The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. *Neuron* 11 (3), 491–502. [PubMed: 8398142]
- (37). Gesemann M, et al. (1996) Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. *Neuron* 16 (4), 755–767. [PubMed: 8607994]
- (38). Campanelli JT, Gayer GG, and Scheller RH (1996) Alternative RNA splicing that determines agrin activity regulates binding to heparin and alpha-dystroglycan. *Development* 122 (5), 1663–1672. [PubMed: 8625852]
- (39). O'Toole JJ, et al. (1996) Alternative splicing of agrin regulates its binding to heparin alpha-dystroglycan, and the cell surface. *Proc. Natl. Acad. Sci. U. S. A* 93 (14), 7369–7374. [PubMed: 8693000]
- (40). Ferns M, et al. (1992) RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. *Neuron* 8 (6), 1079–1086. [PubMed: 1319184]
- (41). Jenniskens GJ, Oosterhof A, Brandwijk R, Veerkamp JH, and van Kuppevelt TH (2000) Heparan sulfate heterogeneity in skeletal muscle basal lamina: demonstration by phage display-derived antibodies. *J. Neurosci* 20 (11), 4099–4111. [PubMed: 10818145]
- (42). Wallace BG (1990) Inhibition of agrin-induced acetylcholine-receptor aggregation by heparin, heparan sulfate, and other polyanions. *J. Neurosci* 10 (11), 3576–3582. [PubMed: 1700081]
- (43). Bowen DC, Gordon H, and Hall ZW (1996) Altered glycosaminoglycan chain structure in a variant of the C2 mouse muscle cell line. *J. Neurochem* 66 (6), 2580–2588. [PubMed: 8632185]
- (44). McDonnell KM, and Grow WA (2004) Reduced glycosaminoglycan sulfation diminishes the agrin signal transduction pathway. *Dev. Neurosci* 26 (1), 1–10. [PubMed: 15509893]
- (45). Mars T, King MP, Miranda AF, and Grubic Z (2000) Heparin blocks functional innervation of cultured human muscle by rat motor nerve. *Pfluegers Arch* 439 (S1), r036–r037. [PubMed: 28176065]
- (46). Yaffe D, and Saxel O (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 270 (5639), 725–727. [PubMed: 563524]
- (47). Gordon H, and Hall ZW (1989) Glycosaminoglycan variants in the C2 muscle cell line. *Dev. Biol* 135 (1), 1–11. [PubMed: 2767331]
- (48). Lin W, et al. (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* 410 (6832), 1057–1064. [PubMed: 11323662]

- (49). Bishop JR, Schuksz M, and Esko JD (2007) Heparan sulfate proteoglycans fine-tune mammalian physiology. *Nature* 446 (7139), 1030–1037. [PubMed: 17460664]
- (50). Oh YI, Sheng G, Chang SK, and Hsieh-Wilson LC (2013) Tailored Glycopolymers as Anticoagulant Heparin Mimetics. *Angew. Chem., Int. Ed* 52 (45), 11796–11799.
- (51). Rawat M, Gama CI, Matson JB, and Hsieh-Wilson LC (2008) Neuroactive chondroitin sulfate glycomimetics. *J. Am. Chem. Soc* 130 (10), 2959–2961. [PubMed: 18275195]
- (52). Huang ML, Smith RA, Triegeer GW, and Godula K (2014) Glycocalyx remodeling with proteoglycan mimetics promotes neural specification in embryonic stem cells. *J. Am. Chem. Soc* 136 (30), 10565–10568. [PubMed: 25019314]
- (53). Rabuka D, Forstner MB, Groves JT, and Bertozzi CR (2008) Noncovalent cell surface engineering: Incorporation of bioactive synthetic glycopolymers into cellular membranes. *J. Am. Chem. Soc* 130 (18), 5947–5953. [PubMed: 18402449]
- (54). Lawrence R, Lu H, Rosenberg RD, Esko JD, and Zhang L (2008) Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans. *Nat. Methods* 5 (4), 291–292. [PubMed: 18376390]
- (55). Agard NJ, Prescher JA, and Bertozzi CR (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition modification of biomolecules in living systems. *J. Am. Chem. Soc* 126 (46), 15046–15047. [PubMed: 15547999]
- (56). Kiessling LL, and Grim JC (2013) Glycopolymer probes of signal transduction. *Chem. Soc. Rev* 42 (10), 4476–4491. [PubMed: 23595539]
- (57). Griffin ME, and Hsieh-Wilson LC (2016) Glycan Engineering for Cell and Developmental Biology. *Cell Chem. Biol* 23 (1), 108–121. [PubMed: 26933739]
- (58). Cornish T, Chi J, Johnson S, Lu Y, and Campanelli JT (1999) Globular domains of agrin are functional units that collaborate to induce acetylcholine receptor clustering. *J. Cell Sci* 112 (Pt8), 1213–1223. [PubMed: 10085256]
- (59). Campanelli JT, Roberds SL, Campbell KP, and Scheller RH (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell* 77 (5), 663–674. [PubMed: 8205616]
- (60). Gee SH, Montanaro F, Lindenbaum MH, and Carbonetto S (1994) Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* 77 (5), 675–686. [PubMed: 8205617]
- (61). Casar JC, et al. (2004) Heparan sulfate proteoglycans are increased during skeletal muscle regeneration: requirement of syndecan-3 for successful fiber formation. *J. Cell Sci* 117 (1), 73–84. [PubMed: 14627628]
- (62). Ghadiali RS, Guimond SE, Turnbull JE, and Pisconti A (2017) Dynamic changes in heparan sulfate during muscle differentiation and ageing regulate myoblast cell fate and FGF2 signalling. *Matrix Biol* 59, 54–68. [PubMed: 27496348]
- (63). Montanaro F, et al. (1998) Laminin and alpha-dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway. *J. Neurosci* 18 (4), 1250–1260. [PubMed: 9454835]
- (64). Migliorini E, et al. (2017) Enhanced biological activity of BMP-2 bound to surface-grafted heparan sulfate. *Adv. Biosys* 1 (4), 1600041.
- (65). Zhang X, et al. (2017) Chemoenzymatic synthesis of heparan sulfate and heparin oligosaccharides and NMR analysis: paving the way to a diverse library for glycobiologists. *Chem. Sci* 8, 7932–7940. [PubMed: 29568440]

Figure 1.

AChR clustering in response to the motoneuron-derived signal agrin, is a hallmark of the formation of the neuromuscular junction. Agrin is regulated by alternative mRNA splicing to include a *B/z* insert that encodes for agrin interactions with its LRP4 receptor and an *A/y* insert containing a KSRK motif recognized by HS GAGs attached to proteoglycan co-receptors at the myotube surface.

Figure 2.

Myotube HS GAGs serve as functional co-receptors for agrin. (A) Binding of exogenous agrin to differentiated mouse myotubes was determined using ELISA. (B) Soluble competitors, heparin and HS, inhibit agrin (10 nM) binding to the myotube surface. (C) The treatment of myotubes with heparinase I–III enzymes (+ hepase) removed cell-surface HS GAG structures (anti-HS antibody 10E4, red) and reduced agrin binding (anti-agrin antibody, green), as evidenced by immunofluorescence microscopy. (D) Cell surface ELISA corroborated loss of agrin (10 nM) binding ability in myotubes treated with heparinases I–III. (E) Agrin (5 nM) triggers the clustering of AChRs, evidenced by punctate spots (green) after staining with AlexaFluor488-bungarotoxin. (F) Attenuation of agrin-mediated AChR clustering through competition with heparin (0.05–50 µg/mL) or the removal of cell surface HS through heparinase enzymes.

Figure 3.

Moderately charged *N*-, 2-*O*-, and 6-*O*-sulfated regions in HS oligosaccharides provide high-affinity binding sites for agrin. Agrin (50 nM) displays minimal binding to nonsulfated oligomers (HS1–6), regardless of length. *N*-Sulfation imparts a marginal increase in binding (HS7–12) and improves with increasing numbers of 6-*O*-sulfation sites (HS13–16) or the introduction of a single 2-*O*-sulfate (H17 and 20). High charge densities in HS oligosaccharides containing additional 2-*O*- or 3-*O*-sulfates attenuate agrin binding (HS18–19 and HS21–24).

Figure 4. Synthetic HS-GAG mimetic glycopolymers serve as agrin co-receptors at the myotube surface promoting AChR clustering. (A) Azide (**1**), biotin (**2**), or phospholipid (**3**) end-functionalized HS mimetic polymers bearing a D2A6 disaccharide (**a**) or a GlcNAc6S monosaccharide (**b**) were generated using RAFT polymerization. (B) SPR analysis indicated high affinity ($K_{D,app} = 6.0$ nM) of mimetic **2a** to surface-immobilized agrin. (C) HS mimetics functionalized with a phospholipid anchoring unit (**3**) were incorporated into the myotube glycocalyx. (D) Only the agrin-binding mimetic **3a** promoted AChR clustering in myotubes cleared of native HS GAG structures by heparinase I–III (hepase) treatment.