DEVELOPMENT OF AN INDUCIBLE, CELL-BASED EXPRESSION SYSTEM TO INVESTIGATE HUMAN POLYSIALIC ACID

by

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(Under the Direction of J. Michael Pierce)

ABSTRACT

Polysialic acid (polySia) is an oncofetal glycan that consists of α2,8-linked sialic acid monomers. This anionic homopolymer is best known as being synthesized on the glycoprotein neural cell adhesion molecule (NCAM). Biosynthesis of polySia in eukaryotes is performed by two polysialyltransferases (polySTs), St8Sia2 or St8Sia4, which are capable of synthesizing chain lengths estimated to contain up to 400 monomers. The interaction between the polyST and its NCAM acceptor, as well as the cellular functions of polySia, have been well-studied. However, how chain length and total polySia production is regulated is still poorly understood. In this dissertation, the hypothesis that changes in polyST expression levels can regulate polySia synthesis was investigated. To this end, a novel, cell-based system that allowed for tetracycline-inducible expression of either St8Sia2 or St8Sia4 in the same cell type was developed. As expected, polyST transcript levels directly correlated with polySia production. Interestingly, however, transcript levels within the range tested had no effect on the polysialylated NCAM glycoforms synthesized, determined by an assay that separated cell lysates using a low percentage polyacrylamide gel, followed by anti-polySia antibody

Western blotting. Moreover, polysialylated NCAM glycoforms synthesized by St8Sia4-expressing cells showed a population that migrated slower than corresponding polysiaylated NCAM glycoforms synthesized by St8Sia2 expressing cells. This result is consistent with the conclusion that the two enzymes show an asymmetry in polySia production. Utilizing the inducible system, cell-matrix adherence assays revealed that increasing polySia expression on NCAM by both polySTs led to a decrease in cell adhesion to both fibronectin and laminin. When compared to cells expressing St8Sia4, St8Sia2-expressing cells resulted in a greater inhibition of cell adherence to laminin; however, these differences between the polySTs were not seen with the fibronectin binding. In conclusion, this inducible system of polyST and polySia has shown to be effective in studying how polyST transcript levels regulate polySia biosynthesis and function in regulating cell-matrix adherence. This system has revealed differences between St8Sia2 and St8Sia4 polysialylation of NCAM in vivo, as well as differential effects of cell adhesion to laminin, effects that have not been reported previously.

INDEX WORDS: POLYSIALIC ACID, TET-INDUCIBLE SYSTEM, CHAIN LENGTH, NCAM, POLYSIALYLTRANSFERASE, CELL ADHERENCE, LAMININ

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DEDICATION

I would like to dedicate this dissertation to my parents, Don and Katie. My parents have always been there to support me in everything I do and have also instilled a value of hard work in me. I would also thank my wife, Mimi, for being there with many words of encouragement as I finished my doctoral work.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Post-translational modification of proteins comes in many forms, including the addition of phosphate groups, lipids, and sugars. These modifications can affect protein function, stability, and location within the cell. This review discusses a specific sugar modification of proteins termed polysialic acid (polySia), which consists of a polymer of α2,8-linked sialic acids. Since polysialylation is added onto N- and O-glycans, a background of both forms of glycosylation will be discussed in this review. The rest of the review will be focused on polySia from biosynthesis, its effect on cell function, and finally its uses in medicine.

HUMAN GLYCOSYLATION

Glycosylation, or the process of co- or post-translationally modifying proteins or lipids with carbohydrates, has been shown to be critical in many cellular processes, such as cell signaling and protein folding. Aberrations in glycosylation cause many diseases and disorders including those that are congenital (1-5). In humans, complex carbohydrates, also referred to as *glycans*, consist of multiple arrangements made from up to ten different monosaccharide building blocks through various linkages with each other forming a saccharide structure, which can be attached to a protein or a lipid. These monosaccharides are: sialic acid, galactose (Gal), glucose (Glc), N-acetylglucosamine

(GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), glucuronic acid (GlcA), iduronic acid (IdoA), mannose (Man), and xylose (Xyl) (4). Unlike DNA replication, RNA transcription, and protein translation, glycosylation is not a template driven process. This process of "building" glycan structures is performed by glycosyltransferases (GTs) that transfer a sugar that is attached to a nucleotide or lipid donor to an acceptor substrate, which could be a protein, glycan, or lipid. The human genome encodes for over 200 GTs (6), which can synthesize an estimated >7000 different glycan structures (7). Although this review will mention the GTs located in the endoplasmic reticulum (ER) where N-glycosylation starts, much of the focus of this review will be on the sialyltransferases and polysialyltransferases that are located in the Golgi and add sialic acid to the ends of glycans.

After synthesis, a majority of sialylated and polysialylated glycoproteins or glycolipids reside at the cell surface forming a structural network termed the *glycocalyx*. Some of the important roles of the *glycocalyx* are cell signaling, modulating protein retention time on the cell surface, as well as recognition by various pathogens (2,4,8). With respect to protein glycosylation, glycans are commonly attached to amine or hydroxyl groups, termed N- or O-glycosylation, respectively (5). Since polySia can be added to both N- and O-glycans, the biosynthesis of both will be described in this review.

Biosynthesis of N-glycans and O-glycans—The mammalian genome is reported to encode over 2500 proteins modified with N-glycans and over 6000 N-glycosylation sites on those proteins as determined by mass spectrometry analysis (9). N-glycans consist of a common core structure covalently linked to an amine side chain of Asparagine (Asn). The N-glycan core is initially constructed on a dolichol phosphate lipid donor in the ER

membrane by a series of GTs producing the pyrophosphate dolichol oligosaccharide structure $Glc_3Man_9GlcNAc_2$-PP-Dol (6). The glycan portion is then transferred *en bloc* onto the protein during translation by an enzyme complex termed oligosaccharide transferase (OST) (10). OST recognizes the consensus sequence N-X-S/T, where X is any amino acid besides proline (10,11) and N is the N-glycan asparagine receptor. After a series of quality control checkpoints to determine if the glycoprotein is folded correctly, the glycoprotein is sent to the Golgi complex through vesicular transport. In the Golgi reside multiple GTs that can further elaborate the core structure (4). Once the glycoprotein transverses through the Golgi from cis-to-trans, it is either transported to a lipid membrane or secreted to outside the cell. There are three categories a mature N-glycan can be classified as: high (oligo-) mannose, complex, and hybrid (**Figure 1.1**). High mannose N-glycans consist of only mannose on their outer antennae, whereas complex can contain additional monosaccharides (i.e. GlcNAc, Gal, Fuc, and sialic acid) on the outer antennae; hybrid structures are a mixture of both high mannose and complex structures on the outer antennae. Additionally, complex structures can consist of two to four antennae that can be terminated by the addition of a sialic acid or polySia.

Unlike N-glycosylation that begins in the ER and then continues to be modified in the Golgi, O-glycosylation occurs predominately in the Golgi, although it should be noted there are forms of O-glycosylation that occur in the cytosol where the glycan (i.e. GlcNAc) can be dynamically added and removed (4). Other differences between these two types of mammalian glycosylation include the initial sugar added to the peptide backbone and the protein side chains to which the glycan is added. With O-linked glycans, Fuc, Glc, Man, GlcNAc, and GalNAc can also initiate glycosylation on Ser, Thr,

and hydroxylysine side chains, whereas N-linked glycans contain only a GlcNAc on an Asn side chain as part of the larger "core" N-linked structure (4,7). Since polySia addition has only been observed on O-linked structures starting with a GalNAc, only this form of O-glycosylation will be discussed further (12). The human genome contains 20 GTs that catalyze the addition of a GalNAc to either a Ser or Thr side chain, this structure is termed Tn antigen (6). Although these GTs catalyze the same reaction, recent work from the Clausen and Gerken laboratories suggest there is a high degree of substrate specificity among these GTs (13,14). The addition of a single GalNAc to Ser/Thr can be further extended by adding a Gal through a β1-3 linkage to form a structure termed a Thomsen-Friedenreich antigen or T antigen. Unlike the addition of the initiating GalNAc where there are 20 GTs that can perform the reaction, the Gal can only be added by one GT known as T synthase. For proper folding of T synthase, a chaperone protein (COSMC) needs to be expressed by the cell. Without COSMC, T synthase is catalytically inactive (15). Similar to N-linked structures, there is a great diversity of O-linked structures as well (7).

For both N- and O-glycosylation, sialic addition occurs at the terminal end of the glycans. This addition of sialic acid is necessary to "prime" the synthesis of polySia for both forms of glycosylation.

Polysialylated N- and O-glycans—Multiple studies have been performed to determine the N-glycans that are modified by polySia. In these studies, polySia substrates from brain lysates were enriched by immunoprecipitation by an anti-polySia antibody. Before analysis of the N-linked structures, these enriched fractions were treated with both peptide:N-glycosidase F (PNGase F), which releases N-glycans from

the glycoprotein, and endoneuraminidase (EndoN) that specifically cleaves polySia (16-19). This latter step of EndoN treatment is necessary to determine the underlining N-linked structure as well as helping with ionization for mass spectrometry analysis. The polySia N-linked structures reported ranged from di-, tri-, and tetraantennary with fucosylation on the chitobiose core (16-20) (**Figure 1.2**). A similar study was performed on O-linked structures except release of the O-glycans was done by β-elimination. The data showed that polysialylation of O-glycans occurs on di-sialylated core 1 or mono-/di-sialylated core 2 structures (12) (**Figure 1.3**). For both N- and O-linked polysialylation, it was not determined if the polysialylation can occur multiple times on the same protein. However, mass spectrometry data from embryonic chick brain experiments suggested that polySia chains can be added to multiple N-glycan antennae (20).

Attempts have been made to determine if core α1,6 fucosylation is critical for polysialylation to occur on N-glycans. To study core fucosylation, Lec13 Chinese Hamster Ovarian (CHO) cells were used. These cells were chosen since they are unable to make GDP-fucose, thus core fucosylation cannot occur (21). In their study, Kojima et al. transfected both Lec13 and wildtype CHO cells with both of the polysialyltransferases, then polySia synthesis was determined by a "chase" assay using radiolabeled glucosamine to metabolically label sialic acid, followed by anti-polySia antibody immunoprecipitation of the lysate. Analysis of the immunoprecipitated lysate was then done by measuring the amount of radioactivity after immunoprecipitation or HPLC of radio-labeled polySia glycopeptides. Using either technique, sialic acid incorporation in the Lec13 cells was below fifteen percent of wildtype cell levels. This phenotype was partially rescued to around thirty percent of wildtype by the addition of

fucose to the media. They also noted that after EndoN treatment, polySia production in wildtype cells recovered more quickly than with the Lec13 cells. Based on these results, it was suggested that core fucosylation is important for polySia N-glycan stability and polySia production (22,23). Although the N-glycan structures that are polysialylated could play a role in its addition, the rest of the review focuses on polySia and its building block sialic acid.

SIALIC ACID AND POLYSIALIC ACID

Sialic Acid is an α-keto-containing nine carbon sugar that is negatively charged at physiological pH (24,25) (**Figure 1.4**). It was independently discovered by two laboratories, first in bovine saliva, then in neural tissue where the groups named the sugar sialic or neuraminic acid, respectively (26-28). It was later established that both sialic and neuraminic acid were the same sugar (24,28). Around twenty years after the discovery of sialic acid, its nine carbon structure was determined (29). The first structure was N-acetylneuraminic acid (Neu5Ac), later two other sialic acid family members were discovered in eukaryotes, N-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxynononic acid (KDN) (24). These three sugars differ at carbon 5, where KDN has only a hydroxyl group and Neu5Ac and Neu5Gc both have N-acetyl groups with Neu5Gc having the acetyl group that is hydroxylated (**Figure 1.4**). All of these structures serve as backbones that can be further modified through acetate, lactate, sulfate, phosphate, and methyl groups. These modifications are so prevalent that there is predicted over 50 distinct sialic acid structures in vertebrates. Unfortunately, most of these modifications are chemically labile; therefore, little is known about their biosynthesis and function

(24,30).

As stated above, eukaryotes can synthesize Neu5Ac, Neu5Gc, and KDN; however, humans can only make Neu5Ac and KDN. This is due to humans having a mutation in the coding region of the enzyme CMP-sialic acid hydroxylase (CMAH) that hydroxylates the N-acetyl group on C-5 on Neu5Ac (31,32). Varki termed the loss of Neu5Gc during evolution the "sialoquake" and hypothesizes it occurred to protect humans from certain pathogens that bound to Neu5Gc. He postulated that the sialoquake caused two ancillary effects: Neu5Gc is immunogenic in humans and human sialic acid-binding immune proteins (e.g. siglecs) only bind Neu5Ac (33).

Biosynthesis of CMP-Sialic Acid—Interestingly, the biosynthetic pathway for Neu5Ac/Gc differs from KDN. For Neu5Ac/Gc, UDP-GlcNAc is the starting sugar for the synthesis, which is then epimerized to ManNAc and finally through a series of reactions converted into Neu5Ac or if C-5 hydroxylation occurs, Neu5Gc. This pathway is highly conserved from bacteria to humans (24,34). The Roseman lab made a key finding for elucidating this pathway when they determined that sialic acid is formed from ManNAc and pyruvate, not GlcNAc and pyruvate (35). KDN, however, is biosynthetically produced by a *de novo* pathway starting with mannose (36). Even though KDN and Neu5Ac/Gc are independently synthesized both have the same nucleotide donor, cytosine monophosphate (CMP). For Neu5Ac/Gc, the last step to form CMP-sialic acid is the addition of the nucleotide donor, which occurs in the nucleus (24). CMP-Neu5Ac/Gc is then transported into the Golgi by the CMP-sialic acid transporter (CST) that resides in the trans Golgi (37).

Sialic Acid Addition—Sialic acid addition occurs in the *trans* cisternae and *trans*

Golgi network of the Golgi, where both sialyltransferases (STs) and CST reside (38). In these cellular compartments sialic acid is added to the terminal end of N- and O-linked glycans (30). Mammals possess around 20 STs that catalyze linkages of α2,3 or α2,6 with Gal, α2,6 with GalNAc, and α2,8 with Neu5Ac (6,39) (**Figure 1.5**); in all cases sialic acid is linked to the previous sugar at the C-2 position (24). Catalytically, STs differ from most other GTs in that they lack the common DxD motif seen in most GTs. This motif helps coordinate cations for glycan transfer to occur; because of this, STs are metal-independent (40). The reaction of adding the CMP-sialic acid to the sugar acceptor substrate is through an SN2-like reaction, where nucleophilic attack is from an OH group of the acceptor to the C-2 of the sugar nucleotide donor. To catalyze this reaction a catalytic base on the enzyme, in the case of the polysialyltransferase it is a histidine, deprotonates this OH group. Before addition onto the acceptor substrate, the sialic acid forms a transition intermediate termed oxocarbenium-ion. This reaction causes the linkage of sialic acid to invert from β on CMP to α on the glycan acceptor (40-42).

polySia History—Unlike the other sialic acid linkages, α2,8 linked sialic acid can be added in a processive manner by specific STs to form a polymer of up to 400 monomers, termed polySia (43). This large anionic polymer was originally discovered in 1957 by Barry and Goebel in *Escherichia coli* K235, which they called colominic acid *(44)*. Although the sugar composition and linkages of colominic acid were not known until 1964, when McGuire and Binkley determined that it was an α2,8 sialic acid polymer (45). PolySia structures in higher organisms were not found until 1978, when they were detected on *Salmonidae* eggs (46). Five years following its discovery on salmon eggs, polySia was discovered in the developing rat brain on the protein Neural

Cell Adhesion Molecule (NCAM) (47). Since then, many organisms have been found to produce polySia, which is usually α2,8 linkages of sialic acid; however, there have been examples of polySia polymers with different linkages, specifically in echinoderms (i.e. sea urchins) and some bacteria strains. Sea urchins have α2,5 linked Neu5Gc polySia on their egg cell surface, where the C-5 hydroxyl group forms the glycan linkage (48). Different bacterial strains can have varying linkages with *Neisseria meningitides* producing α2,9 linked structures while *E. coli* K92 synthesizes a structure that has alternating α2,8/9 linked structures (34). Using molecular modeling, it is predicted that these varying linkages can lead to different secondary structures, with α2,8 and α2,5 linkages forming alpha helical polymers and α2,9, a linear polymer (30). The next few sections will discuss what is known about polySia biosynthesis in bacteria and mammals.

BIOSYNTHESIS OF POLYSIALIC ACID

Biosynthesis of polySia in Bacteria—While the biosynthetic pathway for Neu5Ac/Gc production is quite similar between bacteria and mammals, how polySia is added and the acceptor substrate differ greatly between the two. This is partially due to the difference in the architecture for glycosylation, where bacteria do not possess an ER or Golgi, so a majority of its glycosylation occurs at the cell membrane and wall (34). There does seem to be a high amount of conservation with the polySia synthetic pathway among different bacterial strains, even though these strains have the ability to produce different linkages, as stated above (34,49). In all known pathways, biosynthesis starts at the cell membrane facing the cytosol and ends with polySia being transported to the cell surface (34,49,50). The first step in this pathway is synthesis of the lipid carrier. This

carrier consists of lysophosphatidyl glycerol lipid that has 3-deoxy-D-manno-2-octulosonic acid (KDO) monomers added to it (51,52). Lipid carriers can have up to nine KDO β-linked monomers, although only two to three KDO monomers are necessary to serve as an acceptor for polySia production (53-55). Following elongation, the polySia strand is transported through the periplasm to the outer cell surface (34,49). For *E. coli* the kps and neu genes perform all these steps (including sialic acid production), while *N. meningitidis* has orthologous genes termed sia, ctr, and lip (34).

While the biosynthetic process is highly conserved among different bacteria species, their polysialyltransferases differ in both sequence and biochemical properties. The sequence identity between *E. coli* polysialyltransferase (neuS) and *N. meningitidis* polysialyltransferases (siaD) is only 33% (34). In 2003, Steenbergen and Vimr suggested that neuS and siaD add sialic acid in a processive manner during chain elongation (49). This proposal was challenged in 2007, when the Stummeyer laboratory analyzed siaD in *N. meningitidis* Group B and suggested that it did not follow a processive manner but a distributive manner (53). Unfortunately, the crystal structure of *Mannheimia haemolytica* serotype 2 polysialyltransferase (synthesizes α2,8), did not help answer this question. It did, however, answer two other questions: how the catalytic site adds sialic acid to the growing chain and how the growing chain is retained by the polysialyltransferase. By studying the catalytic residues it was postulated that *Mannheimia haemolytica* serotype 2 polysialyltransferase was an inverting enzyme meaning that sialic acid is being added by an SN2 reaction to the terminal sialic acid, which is common for human sialyltransferases as well (40,50,56). To answer the latter question, an electropositive groove between the two Rossman fold domains was observed to bind the anionic chain (50).

polySia Biosynthesis in Mammals—There are eight ST8Sia family members expressed in mammals (**Table 1.1**). Of these eight family members only two, ST8Sia2 and ST8Sia4, can synthesize polySia on proteins. Both of these polysialyltransferases reside in the Golgi (38). They possess around 60% sequence identity to each other, but vary greatly from the bacterial polysialyltransferases in both structure and glycan substrates (57). Based on the X-ray crystal structure of oligosialyltransferase St8Sia3 and modeling data, human polysialyltransferases possess a single Rossmann domain (GT-A fold) that coordinates nucleotide binding and addition of sialic acid to a protein substrate, while bacterial polysialyltransferases possess two Rossmann domains (GT-B fold) (42,50,56,58). Although structurally these polysialyltransferase differ, both human and bacterial polysialyltransferases are inverting enzymes (40,50).

St8Sia2 and 4 are both modular proteins with canonical domains of sialyltransferases: sialylmotif small (SMS), sialylmotif large (SML), sialylmotif very small (SMVS), and motif III (40,42,57) (**Figure 1.6**). Roles of these domains range from catalysis to substrate and sugar-nucleotide binding. Both SML and SMS domains bind to CMP-Sia, while SMS is also predicted to bind substrate and the SMVS domain is critical for enzyme activity (42,59). The function of the motif III is still unknown, however, it is hypothesized to be important for linkage specificity (42). Another important part of the enzyme's architecture is a pair of disulfide bridges that bring the SMS and SML domains in proximity of each other (40). In addition to the canonical sialyltransferase domains, St8Sia2 and 4 also possess two polysialyltransferase-specific domains: polysialyltransferase domain (PSTD) and polybasic region (PBR). Recent work from the Colley laboratory determined that the PBR domain interacts with the

polysialyltransferases' main acceptor substrate, NCAM (60). While the function of PSTD has not been completely defined, however, it is known through mutational screens that it is important for polysialylation of NCAM (42,61). Using the crystal structure of St8Sia3, which is an oligosialyltransferase with high homology to St8Sia 2 and 4, the C-terminus of the PSTD is located within the enzyme active site while the rest of the domain is an α-helical structure projecting away from the active site (56). Mutations to positively-charged residues in the C-terminus and N-terminus (more distal to the active site) both led to decreased polysialyltransferase activity (61). The residues in the C-terminus are known to bind to the growing polySia chain, while the importance of the N-terminus of the PSTD is still unknown.

To date, only eleven proteins that receive the polySia addition (acceptors) have been identified in mammals, and they can have polySia attached to N-glycans, O-glycans, or both (**Table 1.2**). A hierarchy for polySia substrates has been proposed in a recent review from Schnaar *et al*. (62). Here, the authors hypothesized that NCAM is a preferred substrate for both polysialyltransferases, due to it being the predominant polySia glycoprotein found in humans, and both St8Sia2 and St8Sia4 are capable of polysialylating NCAM. The other polySia acceptors, on the other hand, are polysialylated by only one of the polysialyltransferases (**Table 1.2**).

Work from the Colley laboratory has elucidated in vitro the specificity of the polysialyltransferases toward three of their substrates, NCAM and neuropillin (NRP)-1 and 2. These substrates differ by the fact that polySia on NCAM is N-linked and can be added by both St8Sia2 and 4, whereas polySia on NRP-1/2 is O-linked and is added by only St8Sia4 (**Table 1.2**). The NCAM extracellular domain contains 5 Ig domains and 2

Fibronectin type III domains, with the 6 N-glycosylation sites on the Ig domains (**Figure 1.7**) (63). Of the six N-glycosylation sites only two are able to be polysialylated, both of these sites are located on the fifth Ig domain and are the last two sites of N-glycosylation (i.e. sites 5 and 6). Peptide analysis after polySia immunoprecipitation demonstrated that both polysialyltransferases prefer to transfer to the final/6th N-linked site compared to the 5th site (64-66). By using truncated constructs and mutagenesis of residues, it was determined that the first fibronectin type III domain (FN1) was critical for NCAM polysialylation (63,67). Interestingly, expression of the NCAM FN1 domain alone led to polySia being added to a new O-linked site within the FN1 domain (67). Bhide *et al*. recently showed that FN1 domain directly interacts with the PBR domain of the polysialyltransferases (60). Unlike NCAM, NRP-1 and 2 do not possess Ig or fibronectin type III domains. To test which sites were important for polysialylation of NRP-1/2, plasmids that expressed various domains for NRP-2 were created. These plasmids were then transiently transfected into COS-1 cells and polysialylation was determined using an anti-polySia antibody western blot. The results showed that the merpin A5 antigen-μ tyrosine phosphatase (MAM) domain of NRP-2 is necessary for St8Sia4 binding to NRP-1/2 for polysialylation to occur (68). Currently, these are the only two detailed structural studies of polySia acceptor substrate and polysialyltransferase interactions, while the interaction between the other polySia acceptor substrates and the polysialyltransferases has not been determined (**Table 1.2**).

CHAIN LENGTH OF POLYSIALIC ACID

Analysis of polySia Chain Length—Like the interaction of the polysialyltransferases with their protein acceptors, how polySia chain length is regulated is poorly understood. Before discussing the hypotheses of chain regulation, a background is needed concerning the assays used to measure the chain length. A standardized assay to measure chain length does not currently exist—this has led to the determination of chain length being very problematic, particularly in eukaryotes.

With prokaryotes (i.e. bacteria), pulse assays with radio-labeled Neu5Ac have been used. Release of polySia from lipid donor in prokaryotes is then accomplished by placing the lipid membrane into pH 5.0 buffer causing polySia to be released. This released polysaccharide then can be analyzed by polyacrylamide gel or chromatography (69,70). More recently, Galuska *et al.* had some success with measuring chain length of colominic acid using MALDI-TOF-MS; unfortunately, they were only able to measure chains up to 27 monomers (71,72).

The issues with measuring chain length of polySia in eukaryotes are: 1) intramolecular hydrolysis of the chain during manipulation and labeling, and 2) release of polySia from its core N- or O-linked structures. Studies from the Varki laboratory demonstrated the longer the chain, the more susceptible it is to intramolecular hydrolysis at near neutral pH (73). This is presumably due to an increase of negative charge which leads to acid hydrolysis of the chain. Unfortunately, most of the assays discussed below are performed at low pH, leading to increased hydrolysis when measuring chain length. To counter this intramolecular hydrolysis, polylactones between the C_9 and C_1 can be formed by either addition of a mild acid (pH 3-4) or strong acid (1M HCl) at cold

temperatures. The polylactones are resistant to acid hydrolysis, and can be removed by the addition of a base (74). Unfortunately, polylactonization has not been shown to be effective in studying polySia synthesized in eukaryotes compared to colominic acid analysis, with only one reported use of polylactonization using polySia-NCAM which showed little increase in longer chains determined (around 40 monomers) (71).

The necessity of polySia chain release is due to the labeling reaction of the small molecule, 1,2-diamino-4,5 methylenedioxybenzene (DMB), that exclusively labels the reducing end of sialic acid and can allow for sialic acid detection in the low femtomole range (75,76). Specifically, DMB derivatizes the α-keto group of C_1 which is inaccessible if the sialic acid is attached to another sugar/reduced. To release the polySia chains from the N-glycan as well as cause ring hydrolysis of sialic acid, which is also necessary for DMB labeling, DMB derivatization is performed in low pH solutions (77) (**Figure 1.8**). Currently, there is not a standardized assay that reduces intramolecular hydrolysis, but many chain length assays have been attempted.

Due to polySia being anionic, most chain measurement techniques use a SAX column with high performance liquid chromatography (HPLC) and fluorescence detection using DMB labeling. The first assay is the C_7/C_9 analysis where the polySia is treated with periodate causing the terminal sialic acid to have C_8 and C_9 hydrolyzed leaving a $(Neu5Ac)_7$ structure, while internal sialic acids are protected from this hydrolysis and still have the common Neu5Ac structure (9 carbons). After periodic acid treatment, the sialic acid was derivatized by DMB and the two species of sialic acid were separated using a reverse phase column. Chain length is estimated based on the ratio of C_7 Neu5Ac to C_9 Neu5Ac (78,79). This assay leads to a high amount of extrapolation

based on this ratio, plus the addition of periodate increases intramolecular hydrolysis. Another type of chain length assay is to perform DMB-derivatization to membrane/protein preparations or polySia-NCAM purified samples (76,80-82). As discussed above, DMB labels the reducing end so this reaction needs to be performed at a low pH to cause hydrolysis of the chain, thus allowing the α-keto group to be exposed for DMB derivatization (77,80). This leads to increased intramolecular hydrolysis, as well as a stochastic process that determines where hydrolysis occurs (**Figure 1.9**). Nakata and Troy tried to eliminate some of the problems with these two assays by releasing the polySia chain from its N-glycan core using the enzyme Endo-β-galactosidase that cleaves between N-acetyl-D-lactosamine (Galβ1-4GlcNAc) leaving the polySia chain with a single Gal (43,83,84). In addition to using Endo-β-galactosidase, they separated the released chains on a SAX column and collected the fractions; These fractions were acid hydrolyzed and then sialic acid monomers were labeled with DMB (43). By separating the chains before DMB labeling, Nakata et al. were able to measure chain lengths >400 monomers whereas the previous DMB labeling techniques only measured chains to 90 monomers (43,80). The problem with this assay is the inefficiency of Endo-β-galactosidase at removing polySia chains from the N-glycan structure due to the need of sulfated glycans for the enzyme to cleave the glycan, which most polySia N-glycan structures do not contain (**Figure 1.2**) (43,81). All of these HPLC techniques have shown to have major deficiencies in measuring chain length and have led to inconsistent results (64,85).

Chain length of polySia in mammals has also been assayed using non-HPLC techniques, although they are less quantitative. The most simplistic of these assays is the

use of antibodies to polySia (≥8 monomers) or oligosialic acid (2-7 monomers) (**Table 1.3**) (30,86). A common technique using antibodies to analyze chain length is to separate cell lysate on a low polyacrylamide gel and then immunoblot the transferred membrane with one of the antibodies (87,88). The antibody mAb 735 is commonly used in these assays, which binds ≥10 sialic acids (89). Chain length is then determined by the binding pattern of the anti-polySia antibody, where bands that migrate slower are assumed to possess more polySia (87,88). The advantage of this assay is that little sample is needed due to mAb 735 having high affinity to polySia (K_D=7 nM) (90), in addition, the polySia chains are not exposed to any low pH conditions which reduces the amount of polySia intramolecular hydrolysis. Unfortunately, interpretation of the result of this assay are difficult since the slower migration can be caused by either an increase of chain length or more initiated chains. Also, unlike the assays described above, results from this experiment are not quantitative, making comparisons qualitative in nature.

The last of the techniques are "chase" assays either using radiolabeled or azide-modified sugars. In these assays a sialic acid precursor, either glucosamine, mannosamine, or N-acetylmannosamine (ManNAc), is added to the media or injected into the mice (91-94). Metabolic radiolabeling has been successful with prokaryotes (69,70) and eukaryotes (94) with measuring chain length by fractionating chains using a SAX column followed by determining radioactivity in each fraction. Currently, azide-modified sugars chase assays have only been used to detect polySia production on the cell surface by a click reaction or Staudinger reaction with an accompanying fluorophore or biotin attached (91-93). To date no studies have been published in which azide-modified sugars were used to measure chain length, although it is a promising technique.

Regulation of Chain Length of polySia—As discussed in the previous section, measuring polySia chain length has been difficult, which has made it challenging to answer the question of how chain length is regulated for both pro- and eukaryotes. In their 1979 paper, the Troy lab measured chain lengths of around 200 degrees of polymerization in *E. coli* strain K-235, although it was not determined what regulated the chain length (70). Until recently, there were two prevailing thoughts on regulation of chain length. The first was loss of affinity of the polysialyltransferases to the acceptor substrate as chain length increases, a system seen in *Streptococcus pneumoniae* and its capsular polysaccharide. The other hypothesis was allosteric regulation of the polysialyltransferases either by a polySia pathway enzyme or an ancillary protein that would cause chain termination (34). Because of work from the Priem and Gerardy-Schahn groups, a new hypothesis was proposed, which is the polysialyltransferases themselves can regulate chain length. The Priem laboratory had genetically engineered a subtype of *E. coli* (K12), which does not endogenously express polySia, with a pathway to synthesize a lipid carrier that can serve as a scaffold for the bacterial polysialyltransferases. Polysialyltransferases from different strains (*E. coli* K1, *E. coli* K92, and *N. meningitidis*) were transfected in the genetically modified K12 cells and chain length analysis for each polysialyltransferase was performed. Their results showed that *N. meningitidis* polysialyltransferases created polymers around 30 monomers while both the polysialyltransferases from *E. coli* had polymers of 10-11 monomers (54). The Gerardy-Schahn laboratory shed light on the possible residues within bacterial polysialyltransferases that could be important for chain length regulation. Random mutagenesis on *N. meningitidis* polysialyltransferase was performed, and then the

mutants were screened based on chain length distribution. A mutation to lysine to glutamine within the electropositive groove caused chain length to be more uniform (all chain lengths are equally distributed from 4 to 20 monomers) compared to the highly variable chain length distribution of the wildtype enzyme (95). Based on the recent studies from the Priem and Gerardy-Schahn laboratories, it would strongly suggest that polySia chain length is regulated by the polysialyltransferase, possibly by residues in the electropositive groove.

Compared to bacterial polysialyltransferases, even less is known about how the chain length is regulated by the eukaryotic polysialyltransferases. This is due to problematic techniques to measure chain lengths of polySia on N- and O-linked structures (discussed above) and conflicting data between *in vitro* and *in vivo* studies. *In vitro* studies using purified recombinantly expressed enzymes demonstrated that St8Sia4 leads to around sixty percent longer chain lengths (30 monomers) than St8Sia2 (59). A similar result was seen with overexpression of the polysialyltransferases in a neuroblastoma cell line, where polySia N-glycans from the St8Sia4 clone eluted later on a SAX column than polySia N-glycans synthesized by St8Sia2 (94). By contrast, *in vivo* studies using polysialyltransferase knockout mice concluded that mice with the genotype $St8Sia2^{-/-}St8Sia4^{+/-}$ showed a decrease in chain length of around ten percent (5 sialic acid monomers) compared to wildtype mice, suggesting St8Sia2 is able to create longer chains (81). Both these in vivo and in vitro data would suggest that polysialyltransferases are involved with polySia chain regulation.

Two possible regions that may be involved with chain regulation within St8Sia2 were elucidated when studying single nucleotide polymorphisms (SNPs) of St8Sia2

discovered in schizophrenic patients (96). Researchers then tested the two schizophrenia mutations in St8Sia2 by making two stable cell lines expressing the St8Sia 2 SNPs that are either located in a catalytic domain (i.e. sialyl motif large) or an undefined region between a catalytic and regulatory domain (i.e. sialyl motif large and PSTD domains, respectively). Using the DMB chain length assay, both mutants were found to have polySia chains that were 10-15 monomers less than wildtype St8Sia 2 (114 monomers) (97,98). Both of these regions need to be further studied in both St8Sia2 and 4 to determine if they are important for chain length regulation.

Another hypothesis suggests the presence of a "cap" added to the end of the chains to prevent elongation, which has been reported in other eukaryotic polySia chains. The first example of this capping sugar was seen in sea urchin eggs where a 9-O-Sulfated Neu5Gc was located at the terminal end of its polySia chain (99). Another example is KDN addition, by a novel KDN glycosyltransferase, in trout eggs. Upon addition of KDN, neither Neu5Ac or KDN could be added to the polySia chains (100). Currently, no capping structures have been identified in mammals or chick, possibly due to longer polySia chains (chain lengths of up to 400 monomers compared to sea urchin of 7 monomers) meaning that a modified sialic acid might be at too low a percentage to be detected. In addition, sialic acid modifications can be very labile, also making their analyses technically challenging (24,99).

The last hypothesis is that transcript levels of polysialyltransferases can dictate chain length. In their study, Seidenfaden et al. tried to answer this question by comparing polysialyltransferase transcript level and polySia chain length assay of several polySia-NCAM-positive cell types. They noticed that an increase in polysialyltransferase mRNA

levels compared to a control polySia-positive neuroblastoma cell line (i.e. SH-SY5Y), especially in St8Sia4 expressing cells, caused a slower migration of NCAM glycoforms seen on low percentage polyacrylamide gels followed by immunoblotting with an anti-polySia antibody. This result would suggest that higher polysialyltransferase expression could increase chain length or initiated chains on NCAM (88). It should be noted that in this study only polysialyltransferase transcript expression was determined; other factors, such as NCAM expression levels, were not studied. The fact these other factors were not studied complicates the interpretation of their findings. In chapter 2 of this dissertation, an inducible, cell-based system was created to test directly if polysialyltransferase transcript levels affect polySia production and chain length. Understanding how polySia chain length is regulated could shed light on the different functional roles that have been reported for polySia, which will be discussed below.

IN VIVO FUNCTIONS OF POLYSIALIC ACID

Many of the demonstrated functions of polySia fall into five categories: regulation of cell-cell adhesion, migration, serving as a reservoir for bound growth factors/signaling molecules, regulating membrane protein functions, and modulating the immune response. In eukaryotes, polySia expression is up-regulated during development and decreases into adulthood, which has been determined both by antibody binding and polysialyltransferase mRNA levels (62,101-103). However, data from the Genotype-Tissue Expression (GTEx) project, which performed RNA-seq on over 700 adult donors looking at multiple tissues, shows that both polysialyltransferases and some transcripts of polySia acceptor glycoproteins (**Table 1.2**) continue to be expressed in brain, lymphocytes, and testis even

in adulthood (**Figure 1.10**) (104). When comparing expression patterns, St8Sia2 and NCAM expression are in the same tissues while St8Sia4 expression coincides with its recently reported polySia substrate CCR7. It should be noted that CCR7 is not polysialylated in St8Sia4 null mice; therefore, it is thought to be only polysialylated by St8Sia4 (105). During human embryonic development, polySia is present in all three germ lineages, and its expression is a sign of early differentiation of human induced pluripotent cells (106). The established roles of polySia in both adult homeostasis and human development will now be discussed.

As the name would suggest, NCAM is important for cell-cell adhesion, specifically NCAM-NCAM interaction (107). In addition to these homotypic interactions, other groups have shown NCAM can bind other cell adhesion molecules such as cadherins, integrins, and the extracellular matrix proteins fibronectin and laminin (108-110). Upon the addition of polySia, all of these interactions can be inhibited, leading to decreased adhesion and cell-cell association. Originally this was thought to be the only function of polySia (111,112). This glycan-dependent inhibition of NCAM homo- or heterotypic protein interactions is hypothesized to be important in epithelial-to-mesenchymal transition (EMT), a process that is critical in development and cancer metastasis (113-115). The purpose of EMT is to convert from static epithelial-like cells to motile mesenchymal-like cells, and polySia allows the mesenchymal cells to detach from their epithelial environment by disruption of cell-cell interactions. Experimentally, this has been shown by adding Transforming Growth Factor β1, a known inducer of EMT, to breast cancer cell lines, which caused an increase in polySia expression (116). With clinical samples, polySia expression is correlated with the progression of some

tumors, where late stage tumors express more polySia (114,115). These cancer types include: pituitary tumors, gliomas, rhabdomyosarcoma, colorectal, lung, and breast cancers (117). Along with cell detachment, EMT is also involved with an increase in cell migration.

A function for PolySia in migration was first suggested in the early 1980s from work in Edelman laboratory when a decrease in sialic acid on NCAM (later determined polySia) after maturation of neurons in chick brain was noticed (118,119). A closer look at polySia in neuronal migration was performed in two studies. The first study analyzed interneuron migration from the subventricular zone to the olfactory bulb, while the other study focused on migration within the medial prefrontal cortex. In these studies, polySia was removed by treating neurons with a specific neuraminidase that hydrolyzes α2,8 sialic acid (EndoN) or by using polysialytransferase knockout mice. Once polySia was removed, neuronal migration was severely abrogated in both studies (120-123). One recent example of polySia being involved with migration outside the brain, was shown in dendritic cells migrating to the lymph node. Here it was initially known that polySia increased binding of a ligand, CCL21, which causes chemotaxis of dendritic cells into lymph nodes (124,125). Later, it was determined that polysialylation occurred on a G-protein-coupled receptor, CCR7, suggested on both N- and O-linked sites, which was previously known to bind to CCL21. In St8Sia4 null mice, CCR7 is not polysialylated, which in turn prevented the dendritic cells from trafficking and innervating into the lymph node (105).

In addition to CCL21, polySia has been shown to bind a small number of other signaling molecules (**Table 1.3**). Two well-studied binding partners of polySia are

Fibroblast growth factor 2 (FGF2) and Brain-derived neurotrophic factor (BDNF). Both of these ligands can form a complex with polySia where polySia serves as a reservoir to protect these signaling molecules from proteolysis (57,126). Binding of FGF2 and BDNF occurs if polySia chain lengths are at least 17 and 12 monomers, respectively (**Table 1.3**), as determined using electrophoretic mobility shift assays and later validated using surface plasmon resonance (SPR) (57,126-128). Other ligands linked to neuronal signaling have been shown to bind polySia; this list includes catecholamines (i.e. dopamine) and Ca^{2+} (**Table 1.3**) (57,129). Changes in polySia chain length could affect binding of the neuronal signaling molecules within the brain, which would explain why SNPs identified in St8Sia2 of schizophrenic patients, that cause shorter polySia chain lengths (discussed above), might contribute to the disease symptoms (97,126,129). This idea was further strengthened when Sato and colleagues administered chlorpromazine, a common drug to treat schizophrenia, to mice and noticed an increase of polySia in certain brain regions determined by anti-polySia Western blots (130). It should be noted, however, that most of these studies of polySia and its binding partners have been done in cultured cells, and little is known if these interactions are of importance within the organism.

Along with binding to several signaling molecules (**Table 1.3**), polySia has been shown to interact with receptors on the cell surface and affect their signaling. Two examples of these receptors are fibroblast growth factor 1R (FGF1R) and glutamate receptor ion channels (iGluR). FGF1R has been shown to bind NCAM, and this interaction can cause signaling of the receptor, as well as displacing FGF binding to the receptor (57). As discussed above FGF2 binds to polySia, in this same study SPR data

showed that the polySia-FGF2 complex could disassociate in the presence of heparan sulfate (HS). In addition, association studies using gel filtration chromatography showed that when polySia bound to FGF2 it prevented its binding to FGF1R. Based on these two results, and since HS is critical for FGF1R activation, it has been hypothesized that FGF2 is passed from polySia to HS on the receptor for signaling (57,128). With iGluR, polySia has been demonstrated to affect ion influx. This ion influx effect has been shown in the two iGluR subtypes, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R) and N-Methyl-D-aspartate receptor (NMDAR) (57,129). For AMPA-R, patch clamping experiments were performed where stimulation of AMPA-R with its endogenous ligand glutamate and polySia or polySia-NCAM caused the receptor to stay in the open form (increased ion diffusion) than the AMPA ligand alone (57,131). By contrast to AMPA-R, polySia is an antagonist of the NMDAR, specifically cis binding to receptors containing a NR2B monomer, and causes a decrease of ion influx (57,129,132). It is hypothesized that polySia might displace glutamate binding to the NMDAR, as demonstrated by low levels of glutamate not initiating polarization of neuronal cells when polySia was present (132). Although currently there are only few specific examples of polySia directly affecting specific membrane proteins, a large polyanionic molecule could cause multiple effects, such as increasing electronegativity at the membrane, lowering the pH, and changing membrane potential (133). All of these properties could have a localized impact on signaling of multiple receptors.

One of the most recent functions linked to polySia is its possible cell protective role in the presence of both pro- and anti-inflammatory molecules (134-136). Two recent studies have examined the effect of the pro-inflammatory bacterial lipopolysaccharide

(LPS) on polysialylation using microglia cells and have drawn distinctive conclusions. The first of these studies is from Sumida *et al.* that demonstrated LPS addition can cause an increase in neuraminidase 1 (Neu1) expression and secretion into the ECM leading to polySia degradation. This action liberates polySia-bound BDNF, allowing for BDNF receptor binding and signaling (134). The other study, Werneburg *et al.,* showed that LPS promotes polySia production and upregulates metalloprotease (MPs) expression. Here, the polySia containing proteins, NRP-2 and ESL-1 are quickly shuttled to the cell membrane and are cleaved by MPs, releasing free polySia glycopeptides into the ECM (136). These groups also reported contrasting results regarding the effect of anti-inflammatory interleukin-4 (IL-4) on polySia production in microglia with the former group suggesting an increase in polySia and the latter claiming it had no significant effect (134,136). Currently, these results using LPS or IL-4 have not been tested in a mouse model to determine if either is physiologically relevant. However, in chronic obstructive pulmonary disease where there is increase in protein expression of interleukin-1β (IL-1β), a pro-inflammatory cytokine, there is also an increase in polySia production within the lungs. In the same study, a lung adenocarcinoma cell line was treated with IL-1β and an increase of polySia expression was seen by anti-polySia Western blot. Lastly, using the same lung adenocarcinoma cells, IL-1β and recombinantly-expressed histones, which is known to cause apoptosis, were added to cells. Here, IL-1β administration caused a ten percent decrease in apoptosis, this result was directly linked to polySia production since the addition of EndoN caused a similar amount of apoptosis as histones added alone (135). All of these studies show an importance of polySia in the immune response, although the underlining purpose for most of these responses still remains unknown.

As could be predicted, altered polySia expression has also been linked to some neurological disorders (114,137). For instance, in Alzheimer's disease (AD), polySia was down-regulated only in the entorhinal cortex (EC) in AD patients (NCAM levels were not studied) by immunohistochemical analysis of human brains. This decrease of polySia in the EC was accompanied with an increase of Tau hyperphosphorylation, a known marker for AD, although it was not determined if these two events were linked (138). Other examples of neurological disorders linked to polySia are schizophrenia (described above), bipolar disorder, autism, Parkinson Disease, and prion diseases, where there have either been SNPs seen in patients or an increase in expression of the polysialyltransferases (97,139,140). Of this list, bipolar disorder is probably the most well studied, where SNPs in both St8Sia2 and NCAM have been detected in bipolar patients—including a previously studied SNP in St8Sia2 that has been linked to reduced gene expression. However, biochemical analysis of polySia-NCAM levels have not been determined in these bipolar patients (140). At present, unfortunately, the biological role of polySia in these neurological disorders remains undefined.

POLYSIALIC ACID IN DEVELOPMENT

Expression of polySia peaks during embryogenesis and decreases quite sharply after birth (102). During embryogenesis, polySia expression can be detected in various tissues, including heart, retina, liver, lung, testis, brain, and kidney via immunohistochemical and Western blot analysis using an anti-polySia antibody. The role of polySia in neurological development has been extensively studied, however the role of polySia in other tissues where it is detected is poorly understood (113,141-146).

As discussed above, polySia expression is critical for interneuron migration from the subventricular zone to the olfactory bulb and proper neuronal connections made throughout the brain (122,147-149). Within the developing heart, polySia expression is detected in cardiomyocytes and neurons involved with the conduction system of the heart, while polySia expression decreases throughout the heart in later stages of development (146,150,151). Although studies understanding polySia's role in liver development have not been performed, recent work on liver regeneration after injury has given insight into polySia's possible role in development. Severe liver injury was first induced by a specific diet which also caused an increase in polySia expression on hepatic progenitor cells (HPCs) in adult mice. These polySia expressing HPCs had the ability to migrate to form a new duct within the injured liver as well as an increase in cell proliferation. After fully differentiating into mature hepatocytes, these cells no longer expressed polySia (152). Interestingly, in polySia or NCAM knockout mice, malformation of any of these organs has not been reported besides the brain (147,148,153,154).

In addition to being highly expressed during organ development, polySia expression is also up-regulated during sperm production and fertilized egg placentation (113,155-158). When studying polySia expression in spermatogenesis of roe deer which mate seasonally, thus sperm production is also seasonal, polySia expression in the spermatogonia and Sertoli cells was up-regulated at the start and end of the spermatogenesis cycle (155). In mammals, polySia-NCAM is expressed by sperm cells and it is thought to be important to help the sperm evade the female's immune system (157). Lastly, sea urchin sperm has α2,9 linked polysialic acid attached to the sperm

flagella and has been suggested to be important in sperm motility (158). With egg placentation, which is a process necessary for the formation of the nutrient/waste exchange system between the mother and the growing fetus, polySia is expressed by the cytotrophoblast (CTB). EndoN treatment of isolated CTBs caused a decrease in cell invasion on Matrigel, leading the authors to hypothesize that polySia could be important for CTBs to embed into the uterine wall (156). A recent study using human induced pluripotent stem cells (hIPSCs) shed light on polySia's possible role during germ layer differentiation. It was noted that polyST expression differed as cells were induced to differentiate down different lineages, where St8Sia2 was expressed in the ectoderm and St8Sia4 in the endoderm and mesoderm. When the St8Sia4 gene was knocked out using the Crispr-CAS system or knocked down using shRNAs, hIPSC induced to differentiate into mesendoderm were unable to differentiate (106). Re-expression of the polysialyltransferase allowed differentiation to proceed in the cells with previously deleted enzyme. This work is the tip of the iceberg as far as demonstrating that polySia expression is required for specific cellular functions during the differentiation process and organ development.

POLYSIALIC ACID USE IN MEDICINE

As discussed above, polySia expression is increased in many cancer types, especially later stages of these cancers. Because of this, drugs have been tested to decrease polySia production in hopes to inhibit cancer metastasis. Currently, drugs using modified forms of mannosamine or CMP or even CMP alone have been moderately successful in preventing polySia production and cell migration in cultured cancer cells

(41,115,159-163). It has been proposed that these small molecules bind within the active site and inhibit the donor substrate (CMP-sialic acid) from accessing the active site to be used in glycan transfer (41). Unfortunately, due to their chemical properties, these drugs have low cell permeability, and they likely affect multiple sialyltransferases, not only the polysialyltransferases (115).

Another way polySia is being used in medicine is by either attaching polySia to a protein/peptide or co-administering polySia with a drug to increase efficacy (164-166). The addition of new N-linked glycosylation sites on existing proteins/peptides has been a common technique in the pharmaceutical field to increase the drug's half-life in circulation (167,168). The addition of polySia has shown to be advantageous for increasing protein stability and half-life, lowering immune response, and/or effect enzymatic activity of the drug (164-166). In testing how polySia can increase a drug's half-life, fluorescein was covalently attached to colominic acid and its levels in the serum were monitored by fluorescence. It was determined that the addition of polySia increased fluorescein's half-life up to 40 hours compared to unconjugated fluorescein (169). Biologics that have utilized polySia for improved efficacy include catalase, insulin, anti-tumor monoclonal antibodies, and asparaginase, to name a few (162,164,165,170). With these proteins, the polySia that is synthesized in *E. coli*, which can reach up to 1700 monomers long, is either added to an amine or thiol group of a protein/peptide. With the amine addition, where and how much polySia added is not as controlled; however, with the thiol group addition the polySia is directed to a sequence tag on the C-terminus of the protein that consists of four glycines and one cystine. It should be noted that both types of addition have led to an increase of the proteins half-life from 2-5 fold as well as a 2-15

fold decrease in blood clearance in mice when tested over an extended time (50-75 hours) (162,164,165). Although in a recent study comparing the two polySia-adding techniques on an anti-tumor monoclonal antibody, the amine addition led to 20-fold reduction in antibody binding to the cells when compared to the more specific labeling using the thiol addition. This result was thought to be caused by spurious addition of polySia to key lysines needed for antibody binding, which did not occur using the thiol addition (162). Recent work in the Aebi laboratory tried to circumvent the chemical process used to add polySia. They did this by using an *E. coli* system that could polysialylate recombinantly expressed proteins. The system uses a previously created *E. coli* strain that is able to add a lactose structure onto N-linked sites. They then expressed a bacterial polysialyltransferase in this system, which was able to polysialylate these N-linked lactose structures (170). This bacterial technique might help streamline the addition of polySia to protein/peptides. Currently, there are multiple polySia-modified proteins/peptides that are on stage 2 or 3 trials (164,166).

The addition of polySia alone has also been effective as a scaffold or an excipient (164,166,171-175), which is where an inert molecule administered with a drug that might help protect the drug or increase its absorption. Zhang et al. tested polySia effectiveness as a scaffold when they transected the spinal cord and administered methylprednisolone (MP), an anti-inflammatory drug, with scaffold polymers of polycaprolactone (PCL) and polySia. After testing multiple iterations of a drug administration (PCL, PCL/MP, PCL/PolySia, and PCL/PolySia/MP), PCL/polySia/MP decreased known inflammatory markers and increased nerve growth within the transected spinal cord compared to the other iterations (171). In another recent study, insulin was mixed with polySia and

protamine nanocapsule, which is known to protect a drug from its acidic surroundings. Next insulin was mixed with the protamine nanocapsule alone or protamine nanocapsule and polySia and subjected to acidic conditions that mirrored the intestine. PolySia was able to increase the retention of insulin within the nanocapsule in the simulated intestinal conditions suggesting that polySia increased the protection of insulin more than the nanocapsule alone (174). In the last study, polySia was modified with a cholesterol derivative at the reducing end and added with micelles and the anti-cancer drug doxorubicin. To test the efficacy of this mixture in vivo, S180 cells, a sarcoma cell line, were injected into mice and the tumors were monitored in the presence or absence of drug which was injected intravenously. Remarkably, the tumor sizes were nearly a third of the volume for the polySia/micelles/doxorubicin treated mice compared to the micelles/doxorubicin or doxorubicin alone treated mice (175). These results, along with similar results from two rheumatoid arthritis studies (172,173), demonstrate polySia as a promising excipient. In fact, in a recent review, the authors suggested that polySia will probably overtake polyethylene glycol (PEG) as the most common polymer in drug delivery. They believe this for two main reasons, 1) polySia is not immunogenic and 2) unlike PEG, polySia can be degraded by the body (166).

THESIS OVERVIEW

In this thesis, the relationship between polySia transcript levels and polySia production was tested. To study this relationship, a novel, tetracycline-inducible, cell-based system was created. This system allowed polysialyltransferase transcripts to be induced, while other factors, including NCAM transcript levels, stayed constant. Using

this system, an increase of transcripts of either polysialyltransferase led to an increase in polySia production, as determined by anti-polySia antibody binding to Western blots after SDS-PAGE. To study chain length, this assay was used on cells lysates where a low percentage polyacrylamide gel was used for separation at low voltage. Results of these experiments led to two conclusions: 1) polysialyltransferase expression (within the range studied) had little to no effect on anti-polySia binding pattern, indicating no effect on polySia chain lengths; 2) a fraction of NCAM glycoforms synthesized by St8Sia4 migrated more slowly than glycoforms produced by St8Sia2. The first conclusion contradicts a previous study that suggested that increases in polysialyltransferase transcript levels increase polySia chain length (88). The second conclusion suggests that St8Sia4 is able to either synthesize longer polySia chains or increase the number of initiated chains compared to St8Sia2, since slower migration on the SDS-PAGE is thought to represent an increase in sialic acid incorporation. After characterization of polySia in these induced cell lines, functional assays for cell adhesion were performed. Here an increase in polySia expression led to a decrease in cell adherence to laminin and fibronectin. Additionally, St8Sia2 expressing cells led to a greater decrease in cell-adhesion on laminin compared to St8Sia4 expressing cells. These results are further explained in Chapter 2, while Chapter 3 focuses on future experiments that will help to better understand these results.

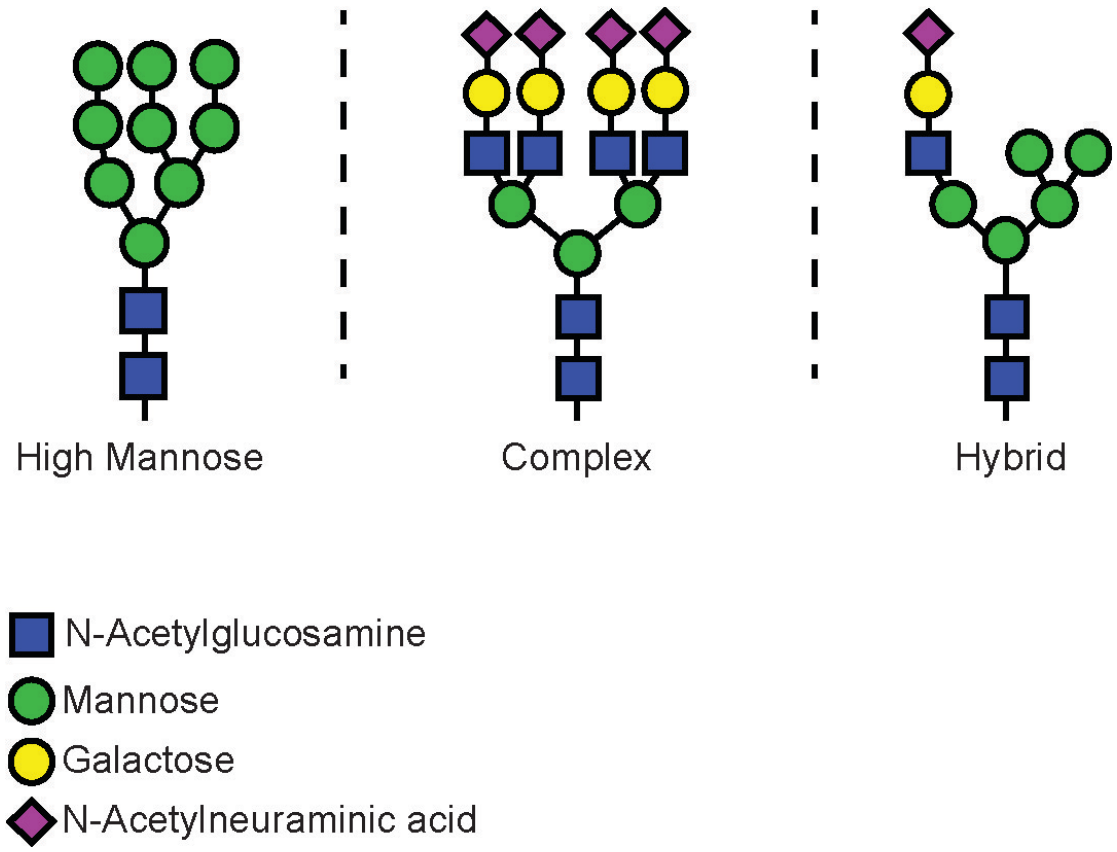

Figure 1.1. Three types of N-glycan structures. These three structures are built on a $Man_3GlcNAc_2$ and attached to an Asparagine side chain. They differ with the outer antennae, where high mannose consists of only mannoses, complex is composed of varying glycans, and hybrid has both a high mannose and complex antennae.

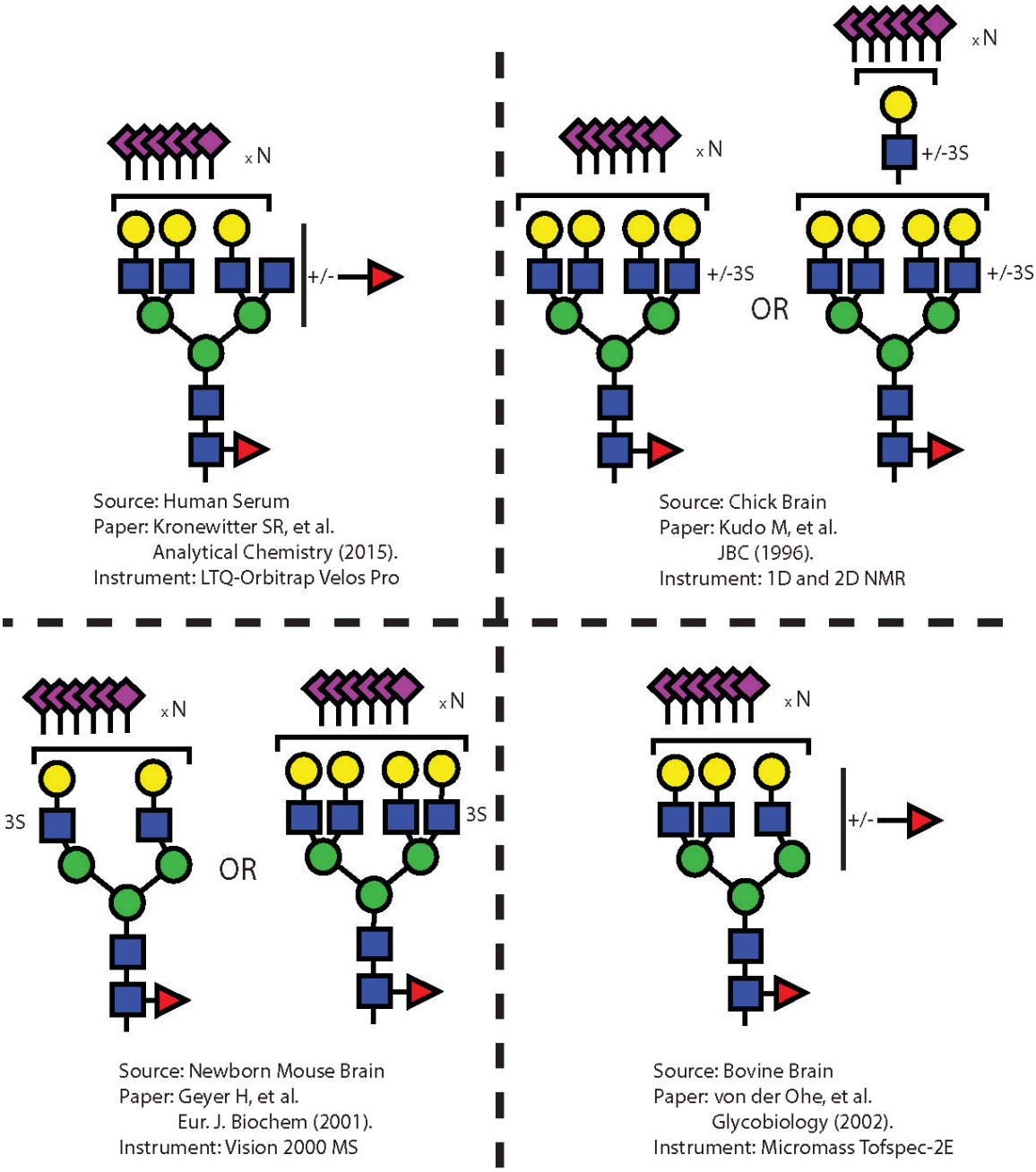

Figure 1.2. Core structures of polysialylated N-glycans. Structures range from di-, tri-, tetra-antennary, but all structures are core fucosylated. The other difference between the structures are sulfation and/or fucosylation to the antennae.

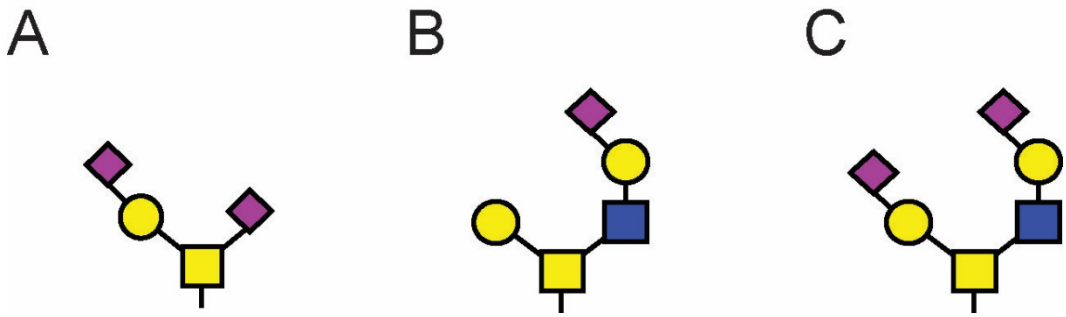

Figure 1.3. Core Structures of polysialylated O-glycans. O-glycan structures on the polysialylated protein NRP-2 were determined using MALDI-TOF, and include di-sialylated core 1 (A), mono-sialylated core 2 (B), and di-sialylated core 2 (C) (12).

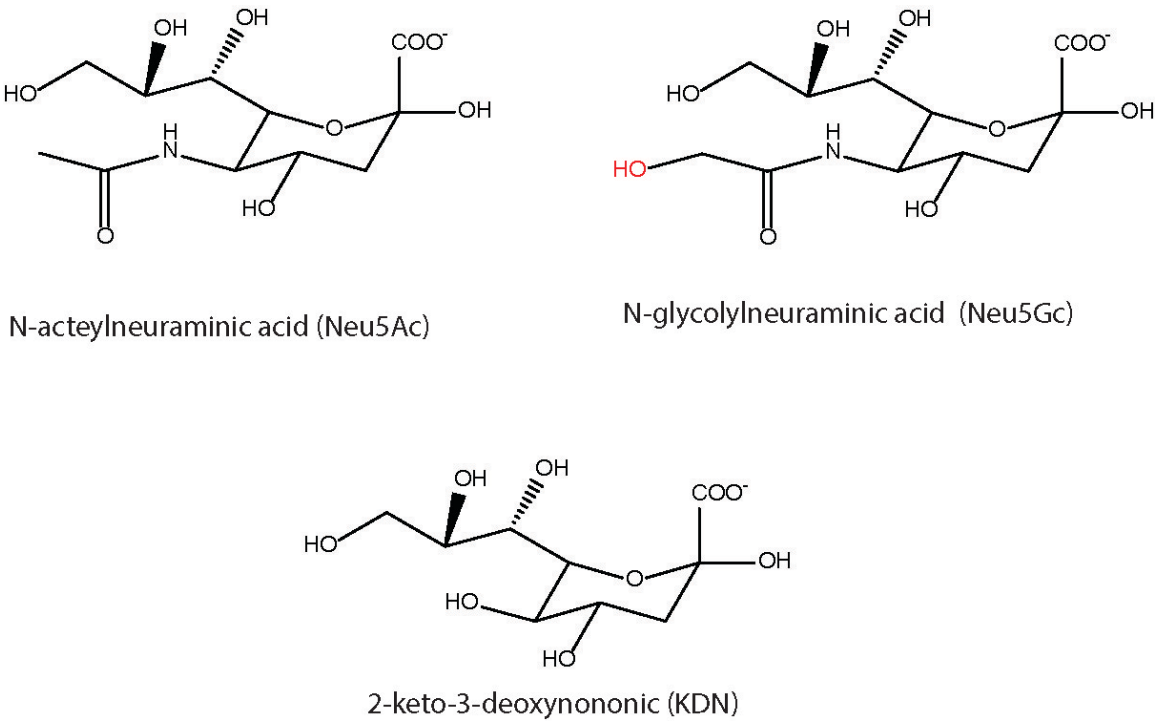

Figure 1.4. Three common sialic acid backbones in mammals. All three structures are nine carbon sugars that have an α-keto group at C1. Humans can only synthesize Neu5Ac and KDN, since humans are null for the hydroxylase needed to add the hydroxyl group (in red) on C5 to make Neu5Gc.

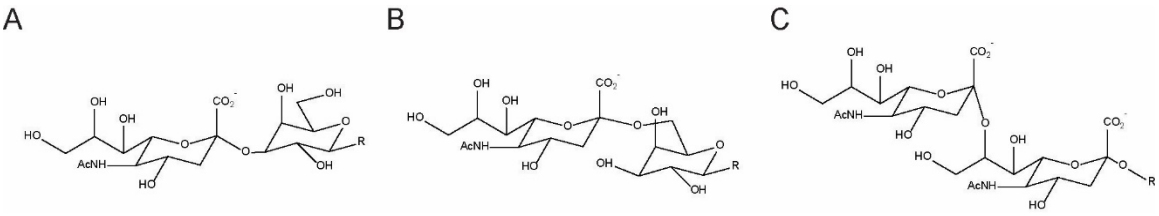

Figure 1.5. Sialic acid linkages in eukaryotes. Linkages consist of (A), sialic acid α2,3 to galactose; (B), sialic acid α2,6 to galactose or GalNAc; (C), sialic acid α2,8 to sialic acid.

Table 1.1. α2,8 sialyltransferase family members in eukaryotes.

α2,8 sialyltransferase	Acceptor	Type of sialylation
St8Sia 1	gangliosphingolipids	di- and tri-sialylation
St8Sia 2	glycoprotein (N-linked)	polysialylation
St8Sia 3	glycolipid and glycoprotein	oligosialylation
St8Sia 4	glycoprotein (N- and O-linked)	polysialylation
St8Sia 5	gangliosphingolipids	di- and tri-sialylation
St8Sia 6	glycoprotein (O-linked)	di-sialylation

Figure 1.6. Human polysialyltransferases functional domains. Although the sequence identity between St8Sia2 and St8Sia4 is only 60%, they are both type II transmembrane proteins containing all the same domains. The canonical sialyltransferase domains are in red, and they are: Sialyl motif large (SML), sialyl motif small, motif III (M3), and sialyl motif very small (SMVS). These domains are critical for CMP-Sia binding (SML and SMS), substrate binding (SMS), linkage specificity (M3), and enzyme activity (SMVS). In blue are the polysialyltransferase specific domains, which include the polybasic region (substrate binding) and polysialyltransferase domain (enzyme activity and possible substrate binding). The transmembrane (TM) domain is in green. This image was adapted from Colley, K. J., Kitajima, K., and Sato, C. (2014) Polysialic acid: biosynthesis, novel functions and applications. Critical reviews in biochemistry and molecular biology 49, 498-532 (61).

Table 1.2. Validated polysialylated glycoproteins.

Substrates	N- or O-linked	St8Sia2 and/or St8Sia4	Where Expressed
NCAM1	N-linked	St8Sia 2 and 4	Neuronal cells and leukocytes
Neuropilin-2	O-linked	St8Sia 4	Dendritic cells
Neuropilin-1	O-linked	St8Sia 4	Dendritic cells
SynCAM 1	N-linked	St8Sia 2	neuronal cells
CCR7	N- and O-linked	St8Sia 4	Leukocytes
CD36	O-linked	N.D.	human milk
sodium channels alpha subunits	N-linked	N.D.	rat brain
potassium channels	N-linked	N.D.	rat brain
St8Sia 4	N-linked	St8Sia 4	within Golgi
St8Sia 2	N-linked	St8Sia 2	within Golgi
E-selectin ligand 1	N.D.	St8Sia 2	Microglia

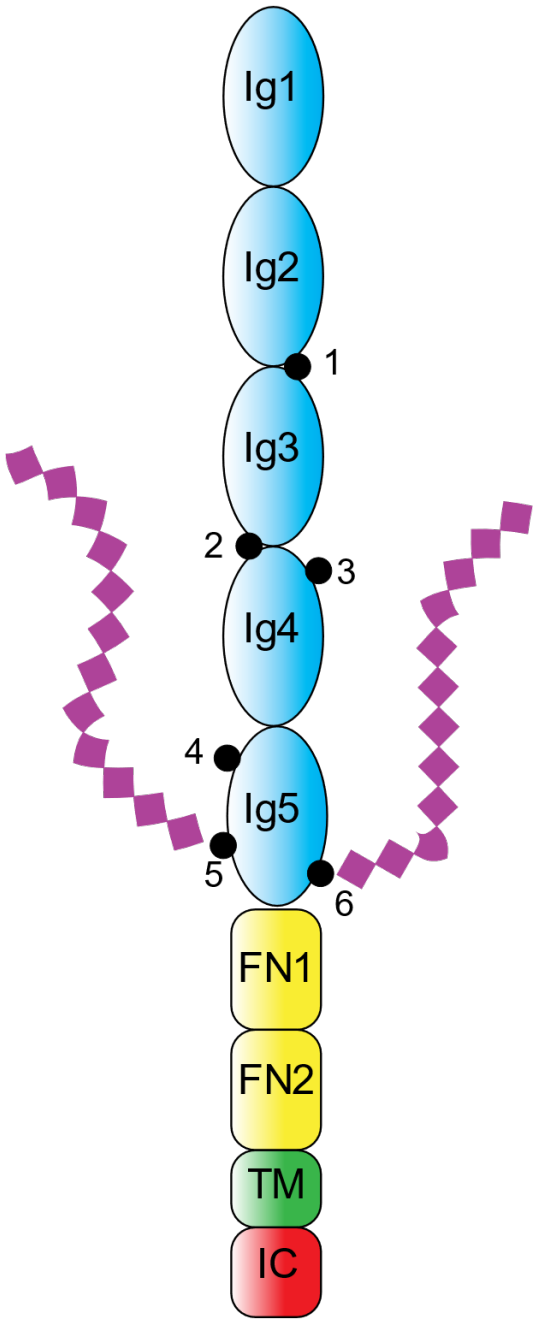

Figure 1.7. NCAM protein architecture. NCAM is a type-I membrane protein that contains two FNIII domains (yellow) and 5 Ig domains (blue). The 6 known N-glycan sites on NCAM are denoted by black circles, while only the 5th and 6th sites can receive

polysialylation, as shown by the purple diamonds. Both NCAM-180 and NCAM-140 also contain transmembrane (TM) and intracellular domains (IC).

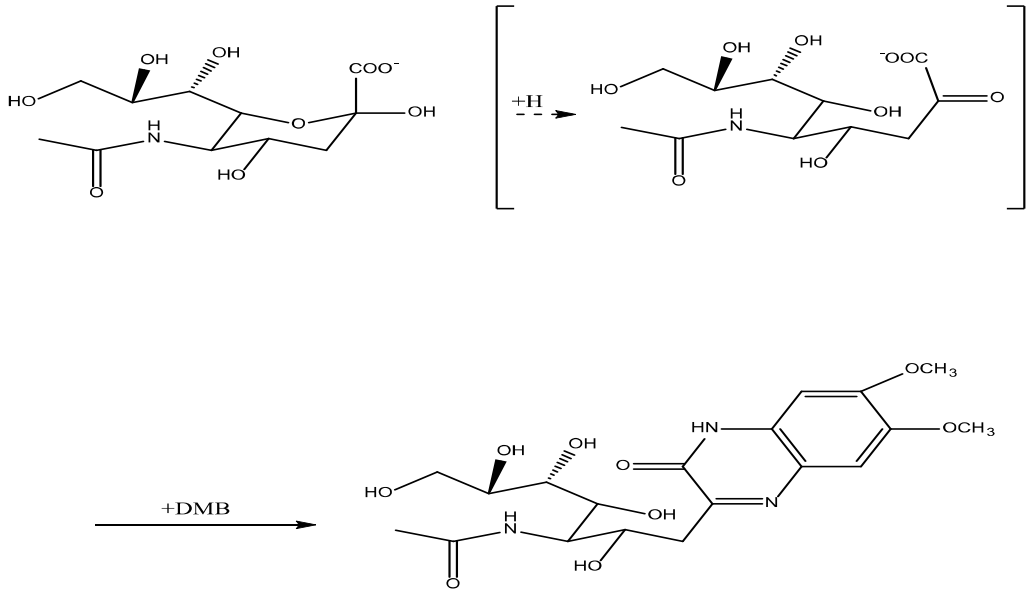

Figure 1.8. Proposed mechanism of DMB derivatization of Neu5Ac. DMB derivatization is performed under acidic conditions which causes a ring cleavage of Neu5Ac. DMB then derivatizes the α-keto group at C-1 of Neu5Ac.

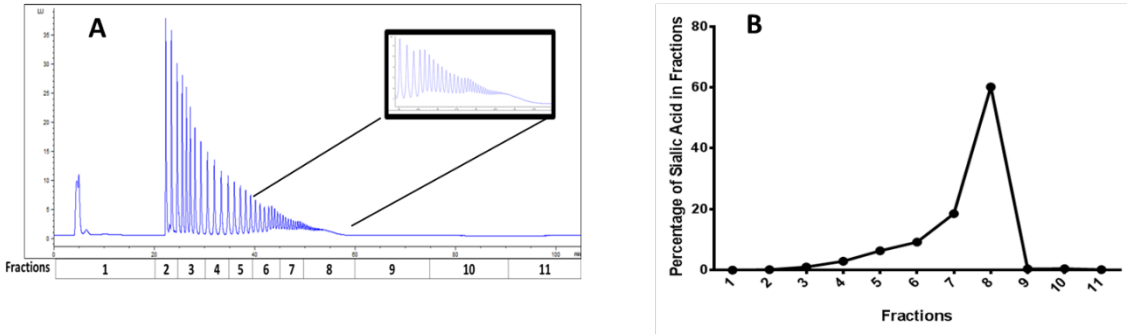

Figure 1.9. Acid hydrolysis of polySia by DMB derivatization. (A) 1 µg of colominic acid was labeled with DMB at 55°C for 2 hours. The labeled sample was then separated on a SAX column with product detection using fluorescence (ex. 373 em. 410). Insert is zoom in from 40 to 60 minutes. (B) 1 µg of unlabeled colominic acid was first fractionated on a SAX column, and fractions pooled as shown on the bottom of figure 1.4A. Fractions were then hydrolyzed with 1M HCL and DMB labeled. Each fraction was then on a C18 column and detected with fluorescence. Percentages are calculated relative to the sum of sialic acid detected in all the fractions.

Table 1.3. Binding partners and antibodies to polySia. The blue group are signaling molecules that bind to polySia, while the red represents antibodies. Degree of polymerization (DP) is the minimum number of sialic acid monomers to bind. Data from reviews (61, 105).

Ligand/Antibody	DP required for binding
BDNF	12
FGF2	17
Dopamine	>2
Epinephrine	n.d.
PDGF	n.d.
CCL21	n.d.
histone H1	n.d.
mAb 735	≥11
H.46	≥8
OL.28	≥4
12F8	n.d.

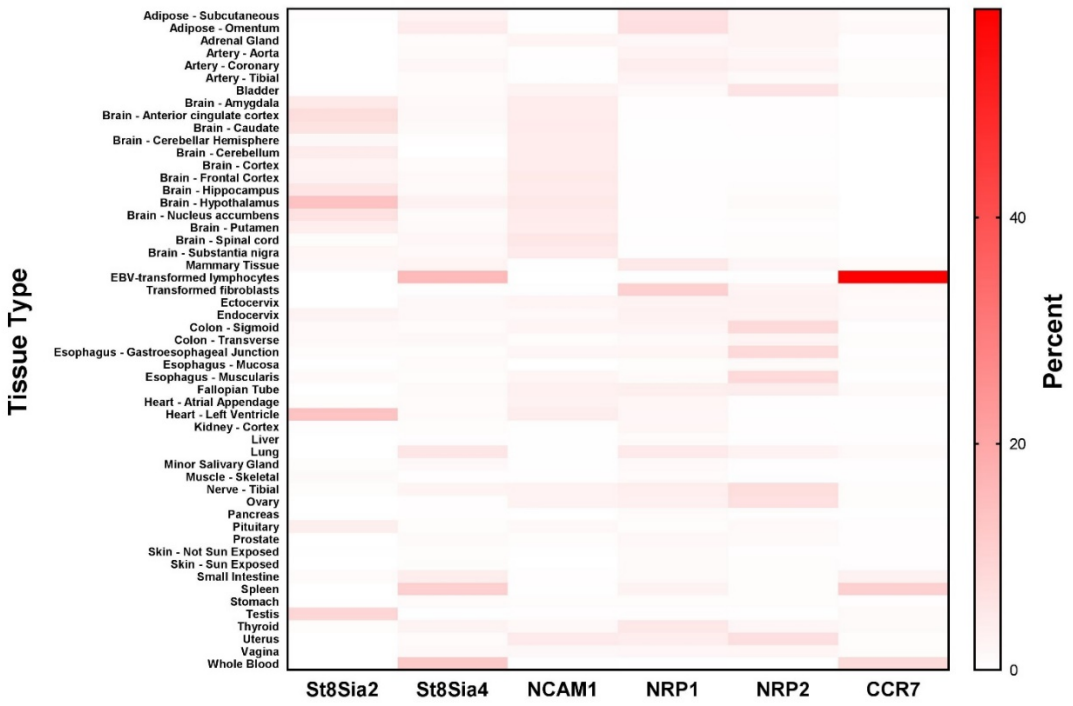

Figure 1.10. mRNA expression of St8Sia2, St8Sia4, and protein acceptors in adult tissue. mRNA values were obtained from Genotype-Tissue Expression (GTEx) project, that analyzes multiple adult tissues using next generation sequencing. Percent of expression was based on the total amount of mRNA expressed in tissue type divided by the total amount mRNA expressed in all the tissues for each respective transcript.

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CHAPTER 2

INDUCED EXPRESSION OF TWO POLYSIALYLTRANSFERSES, ST8SIA2 AND IV, RESULTS IN DIFFERENCES IN POLYSIALYLATED NCAM GLYCOFORM AND CELL-MATRIX ADHESION

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ABSTRACT

Polysialic Acid (polySia) is a homopolymer of α2,8-linked sialic acids attached primarily to the glycoprotein neural cell adhesion molecule (NCAM). NCAM polysialylation is performed by two polysialyltransferases (polySTs), St8Sia2 and 4, which can synthesize polySia either individually or in unison. Studies using various cell types have associated increased polyST transcript levels with increased polySia production and polySia chain length, although other variables between the cell types were not investigated. To compare directly the impact of changes in expression of the two enzymes, we developed a tet-on inducible system that expressed either St8Sia2 or St8Sia4 in the same cell type, thus eliminating cell type heterogeneity as a confounding factor for the interpretation of results. As expected, polyST transcript levels correlated with polySia production, determined by densitometry of anti-polySia antibody Western blot after SDS-PAGE. Strikingly, however, little difference was detected in the polysialyated NCAM glycoforms produced by each polyST clone after increased enzyme induction, even though transcript levels increased by 4-fold. In addition, compared to St8Sia2, increased St8Sia4 expression levels both resulted in decreased adhesion to fibronectin while, by contrast, St8Sia2 showed significantly less inhibition of adhesion to laminin than St8Sia4. These results suggest that St8Sia4 and St8Sia2 do not synthesize stucturally or functionally identical populations of NCAM glycoforms.

INTRODUCTION

Polysialic acid (polySia) is an anionic homopolymer consisting of α2,8 linked sialic acid and can range from 8 to 400 monomers (1,2). This polymer was originally found in *E. coli* K12 and later identified in mammals (i.e. rat brain) by the Finne laboratory (3,4). Mammals can express six sialyltransferases that synthesize α2,8 linked sialic acid; however, only two of them can synthesize polySia (≥8 sialic acids), St8Sia2 and 4 (5,6). These polysialyltransferases (polyST) are highly specific for their protein acceptors that receive this glycan modification, with only 11 proteins identified as yet to contain polysialic acid (5). The predominant acceptor by far for both St8Sia2 and St8Sia4 is the neural cell adhesion molecule, NCAM, and on this acceptor the polySia is attached to N-linked glycans, which is true for the majority of the other acceptor glycoproteins.

St8Sia2 and St8Sia4 are both type II membrane bound glycosyltransferases that reside in the trans-Golgi. Protein sequence identity between the two polySTs is about 60 percent, with both enzymes possessing the same modular domains (5). In general, expression of both polySTs increases during development and reduces after birth and into adulthood (7,8). Expression of these polySTs is cell and tissue specific (7,9-15). For example, neither enzyme is expressed in human embryonic stem cells, but differentiation along the mesendoderm pathway is characterized by induction of St8Sia4 expression. In contrast, St8Sia2 is expressed as cells differentiate along neuroectoderm pathways, due to distinct transcriptional regulation (11).

Due to the anionic properties of polySia, the first function studied for polySia was its inhibition of cell-cell adhesion, speculated to be caused by repulsive negative charges

on adjacent cell surfaces (16-19). This repulsion caused a decrease in homotypic adhesion between NCAM on opposing cell surfaces, as well as adhesion to matrices such as fibronectin and laminin (16,20-23). This anti-adhesive property is critical for polySia's function in neuron migration that occurs during brain development (18,22,24-27).

A major question concerning the biosynthesis of vertebrate polySia regards the mechanism(s) of regulation of its transfer to sialylated N-glycans on NCAM, especially related to the polySia chain length. One possibility is that polyST transcript expression levels regulate polySia chain length—the higher the expression, the longer the chain lengths synthesized. Another is that chain length is regulated by NCAM or other acceptor expression levels. To investigate the association of polyST transcript levels and polySia chain length, St8Sia2 and St8Sia4 transcript levels were compared, along with chain length analysis, for different mammalian cell lines that express polySia on NCAM. These results suggested an association of polySia chain length with polyST transcript levels. Additionally, St8Sia4 expression led to longer polySia chains synthesized than St8Sia2 expression (28). This result was consistent with in vitro studies (29,30), but in vivo studies have suggested that St8Sia2 synthesizes longer chains (31,32). It should be noted that the relative expression levels of NCAM, the sole polySia acceptor in all these cell lines, or other transcripts involved with N-glycosylation, were not determined. Differences in these transcripts would likely affect polysialylation of NCAM, which makes interpretation of these results problematic.

The focus of the present study was to use a single cell type to test the hypothesis that polyST transcript expression levels alone are a primary determinant of polySia length. We hypothesized that low levels of expression would be expected to produce relatively

short lengths of polySia, and higher levels would be expected to produce relatively longer chains. To this end, we developed a tetracycline-ON system in a single cell type such that the transcript expression of each polyST could be separately modulated by the addition of doxycycline (DOX). To eliminate differing acceptor concentrations from being a variable, Neuro2a cells that show high levels of NCAM constitutive expression, but do not express either polyST, were chosen (33).

Several methods are currently in use to measure polySia chain length, but these all require chemical release or derivatization of the polySia chains under acidic conditions, which are known to result in hydrolysis of polySia (34). A simpler technique that is highly reproducible was chosen to visualize patterns of polysialylated NCAM glycoforms and relative chain lengths (28,35,36). This method uses SDS-PAGE to separate glycoproteins in cell lysates, followed by Western blotting using an anti-polySia antibody to detect polySia on glycoprotein acceptors, in this case, NCAM. Differences in maximal chain lengths were then noted by observing the slowest migrating polySia-immunoreactive banding patterns.

Our results show that polyST transcript expression levels regulate the total amount of polySia production, as indicted by relative levels of anti-polySia antibody binding. Interestingly, however, increased expression levels of either polyST had little significant effect on the banding patterns of the polySia-NCAM glycoforms that reflect changes in chain length. In addition, St8Sia4 synthesized a population of polySia that migrated more slowly when compared side-by-side to St8Sia2-synthesized polySia, suggesting an inherent difference between the two enzymes in terms of the synthesis of slower migrating NCAM glycoforms expressing polySia.

PolySia expression by both enzymes led to a decrease in adhesion to both fibronectin and laminin. Given comparable levels of polySia, however, the level of inhibition of adhesion by St8Sia2 was greater than that by St8Sia4 to laminin. Interestingly, this difference was not observed with adhesion to fibronectin. These two results suggest that St8Sia2 and 4 show differences in both their biosynthetic properties, as well as functional differences in their polysialylated products.

MATERIALS AND METHODS

Plasmid Construction— For introducing sequences into the tet-inducible plasmid, the Gateway Cloning system was used. First, the polyST cDNA was cloned into a Gateway entry vector, pEntr4-no ccdB (Addgene Plasmid #17424). The polyST cDNA were from the pCAG-St8Sia2+V5 and pCAG-St8Sia4+V5 plasmids, which were a kind gift from Dr. Stephen Dalton (University of Georgia). pCAG-St8Sia2+V5 or St8Sia4+V5 and pEntr4-no ccdB plasmids were cut simultaneously in the same tube with NotI (Promega, Madison, WI) and NcoI (Promega, Madison, WI) at a ratio of 5:1 (i.e. 100 ng to 20 ng), respectively. The digest was performed for 2 hours at 37°C followed by heating to 80°C for 20 minutes to deactivate the enzymes. From the digestion, 2 µL of the 20 µL reaction were added to ligation solution (Promega, Madison, WI) and incubated at room temperature for 15 minutes. The ligation reaction was added to DH5α cells (Thermo Fisher, Waltham, MA) and the transformation was performed as described by the manufacturer followed by being plated onto kanamycin LB plates. Sequences for pEntr4-St8Sia2+V5 and pEntr4-St8Sia4+V5 were confirmed using Sanger sequencing. Next, the Gateway LR Clonase II Enzyme mix (Thermo Fisher, Waltham, MA) with the

pEntr4 plasmids and the destination plasmid, pCW57.1 (Addgene Plasmid #41393), where added together in one tube. pCW57.1 is a third generation tet-inducible vector that possesses the tet-on response element followed by the gateway entry site and a separate promoter that expresses the reverse tetracycline-controlled transactivator and puromycin resistant genes. The LR clonase reaction was then transformed into NEB Stable competent *E. coli* (New England Biolabs, Ipswich, MA) cells as described by the manufacturers and plated onto ampicillin LB plates. Sequences were then confirmed using Sanger sequencing.

Cell Lines and Maintenance—Neuro2a cell line (ATCC) were maintained in Eagle's Minimum Essential Media (EMEM) with L-glutamine (Lonza Group, Basel, Switzerland) supplemented with 10% FBS. The selected clones were grown in EMEM with 10% FBS and 1 µg/mL puromycin. HEK 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine (Lonza Group, Basel, Switzerland) supplemented with 10% FBS.

Production of Lentivirus—Lentivirus was produced as described by Yang et al. (37). In this protocol, HEK 293T cells were transfected by 2nd generation packing plasmid (kind gift of Dr. Stephen Dalton) and pCW57.1 plasmid either expressing St8Sia2 or St8Sia4. Specifically, 2 X 10^5 HEK 293T cells were plated per well on a six well dish. Cells were grown overnight to 70-80% confluency. The next day, a plasmid mixture of 500 ng of psPAX2, 50 ng of pCMV-VSV-G, and 500 ng of pCW57.1 plasmid were added together in Opti-MEM media (Thermo Fisher, Waltham, MA), along with a solution with 7 µl of Lipofectamine 2000 (Thermo Fisher, Waltham, MA) in Opti-MEM media. Both solutions were incubated at room temperature for 5 minutes. Next these

solutions were combined and incubated at room temperature for 30 minutes. Finally, the combined solution was then added to the cells and incubated for 18 hours at 37°C. After 18 hours, the transfection media was removed and replaced with 2 mL of DMEM supplemented with 20% FBS. Supernatants containing viral particles were harvested and pooled after 24 and 48 hours and spun at 2000 rpms for 10 minutes and filter through a 45 µm cell filter.

Establishing Stable Cell Lines—2.0 mL of supernatant containing viral particles (described above) with polybrene (8 µg/mL) were added to Neuro2A cells (5 X 10^5 cells per well) in a six well dish. Infection media was removed after 24 hours and replaced with fresh media. Ninety-six hours after infection, selection with 3 µg/mL of puromycin was performed; Selection was performed twice for St8Sia2 and 4. To determine if infection was successful, cells were lysed in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP-40, 0.1% SDS, and protease inhibitor cocktail set V, EDTA-free (Millipore, Burlington, MA)) on ice for 30 minutes. Lysate was then centrifuged at 14,000 rpm for 10 minutes and the supernatant was removed. Fifty µg of total protein lysate, determined by Bradford colorimetric assay (Thermo Fisher, Waltham, MA), in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA Laboratories, Hercules, CA) with 5% of beta-mercaptoethanol were incubated at 55°C for 5 minutes. The lysate was then run on a 4-20% Mini-Protean TGX gel (Bio-Rad Laboratories, Hercules, CA) and transferred to PVDF. For protein size determination, Precision Plus Protein Ladder (Bio-Rad Laboratories, Hercules, CA) was used. Blotting was then performed using anti-polySia (mAB 735—Absolute Antibody, Oxford, UK) or anti-β-actin antibodies (Santa Cruz Biotechnology Inc., Dallas, TX) followed by secondary antibodies conjugated with

HRP (Santa Cruz Biotechnology Inc., Dallas, TX). Enhanced chemiluminescence substrate (PerkinElmer, Waltham, MA) was then added to membrane, and visualization was performed using X-ray film (Denville Scientific Inc., Metuchen, New Jersey).

Following selection, FACS was performed using a MoFlo XDP (Beckman Coulter, Brea, CA) in 96 wells based on polySia production. Sorting was performed on uninduced clones and untransfected Neuro2a cells. The untransfected Neuro2a cells served as a negative control for polySia production. Cells were first detached from the plate using 2 mM of EDTA in PBS that was incubated at 37°C for 10 minutes. Detached cells were then washed once using 1X Phosphate-Buffered Saline (PBS) (Lonza Group, Basel, Switzerland) and once with flow cytometry buffer (1X PBS and 1% BSA that was filtered by a 0.45 μm filter). Cells were then incubated with mAb 735 at 1:100 dilution in flow cytometry buffer at 4°C for 30 minutes while rocking. Cells were washed 3 times with PBS and once with flow cytometry buffer. Secondary antibody of Anti-mouse IgG Alexa Fluor 488 (Thermo Fisher, Waltham, MA) was incubated with the cells at 1:100 dilution for 30 minutes at 4°C while rocking. Labeled cells were washed 3 times with PBS and once with flow cytometry buffer. Cells were finally suspended in 500 μL of flow cytometry buffer and run through cell strainer (Thermo Fisher, Waltham, MA). For sorting, Neuro2a labeled cells were analyzed first to help set the gating. The gating was then set to no or low polySia binding followed by sorting of uninduced clones. Three 96 well plates were sorted for each puromycin selected clone (two for each polyST), leading to over 500 clones sorted for each polyST.

Lastly, screening of the clones was performed by dot blot using mAb 735. Specifically, plus or minus DOX (1 μg/mL) (Sigma-Aldrich, St. Louis, MO), a

tetracycline analog, for 72 hours was added to screened clones. Cells were then lysed using 125 µL lysis buffer as described above. 2 µL of lysate was added to nitrocellulose (Bio-Rad Laboratories, Hercules, CA), followed by blotting with mAb 735 and detection by HRP (described above). Densitometry was then performed for each clone using ImageJ (38). Clones were then compared based on least mAb 735 binding of the non-doxycycline induced lysate. The lowest binders (<4% of DOX induced clone) were then selected for both polyST, which we termed ST2D7 and ST4F6 for inducible clones of St8Sia2 and 4, respectively.

qRT-PCR— Control and DOX treated cells were harvested, flash-frozen in liquid nitrogen and stored at -80°C until use. Total RNA isolation and cDNA synthesis on 3 biological replicates was carried out as described previously (Nairn et al, 2007). The qRT-PCR reactions were performed in triplicate for each gene analyzed using primer pairs listed below. Amplification conditions and data analysis was performed as described previously (Nairn et al, 2010). Briefly, Ct values for each gene were normalized to the control gene, RPL4, prior to calculation of relative transcript abundance. The following primers were used: St8Sia2 (forward: 5′-CTCAATGGCAGCATCCTGT-3′, reverse: 5′-CTCGTTGACCCACTCAACAC-3′), St8Sia4 (forward: 5′- GAGGAGAGAAGCACGTGGAG-3′, reverse: 5′-AGGCAGTTCGCACTTTCAGT -3′), and RPL4 (forward: 5′-AGAAGGCTGCTGTTGGTGTT-3′, reverse: 5′- TGGTTTCTTGGTAGCTGCTG -3′). For time course analysis, relative values were normalized to 96 hours minus DOX to show fold change after DOX induction.

Doxycycline Dose Response Assay—Log dilutions of DOX were made ranging 1

ng/mL to 10 µg/mL in selected clone media (described above) and added to cells that are around 80-90% confluent in a six well dish. For a negative control, no DOX was added to the well. After 24 hours, cells were harvested and lysed. The cells were lysed as described above. For all samples 10 µg total lysate protein in Laemmli buffer in 5% of beta-mercaptoethanol were incubated at 55°C for 5 minutes. The lysate was separated on a 4-20% Mini-Protean TGX gel and Western blotted for polySia and actin as described above. Densitometry was performed on the blots using ImageJ. To compare multiple blots the ratio of polySia to actin densitometry values was used.

Time Course Assay—Cells were plated at 5 X 10^5 cells per well in a six well dish and allowed to grow overnight (50-60% confluent). The next day, either 1 µg/mL or 10 µg/mL of DOX were added daily over 96 hours, while samples were taken every 24 hours. The cells were lysed and Western blotted for polySia and actin as the dose response assay (discussed above). Densitometry and analysis for 10 µg/mL was performed as described above. For 1 µg/mL, the ratio of polySia to actin was performed then values were normalized by setting 96 hours plus DOX as 100%, which was the highest polySia to actin ratio for both polyST clones.

Relative Chain length Comparison assay—Chain length analysis was done as described by Seidenfaden et al (28). For the assay we used lysate from the time course using 1 µg/mL DOX for both polyST clones. The amount of lysate loaded was adjusted to have the same amount of polySia loaded for each sample. Lysate was prepared in Laemmli buffer with 5% of beta-mercaptoethanol and then incubated at 55°C for 5 minutes. Prepared lysate and Precision Plus Protein Ladder were loaded to 7.5% Mini-Protean TGX gel and run overnight (~18 hours) at 30 volts. After transferring to PVDF,

a Western blot using mAb 735 was performed (described above).

Binding Assays—Laminin-binding assays were performed similar to Acheson et al. (39). First, 100 µg/mL of poly-L-lysine was added to each well and incubated overnight at 4°C. The next day, poly-L-lysine was removed, and the wells were washed 3X with PBS. Laminin was then added to the poly-L-lysine coated wells at 5µg/mL (Thermo Fisher, Waltham, MA) and incubated at 37°C for 2 hours. For fibronectin coating, 1.5 µg/mL of fibronectin (Trevigen, Gaithersburg, MD) was added to the wells and incubated overnight at 4°C. Fibronectin was then removed and 1X wash was performed using sterile water. Next, blocking was performed using 2% BSA in PBS for 1 hour at 37°C. Adhesion of cells to poly-L-lysine-coated wells was measured as a control. Both St8Sia2 and 4 cells adhered similarly to polylysine wells; the extent of adhesion after 15 min. was significantly less than to either fibronectin or laminin.

For all binding assays, cells were similarly prepared and diluted to give 7.5 X 10^4 cells per well. Cells were first plated and then induced with (1µg/mL or 10µg/mL) or without DOX. After 24 hours, cells were washed 1X with PBS then removed from the plate using 125µM of EDTA for 3 minutes at 37°C. These cells were then pelleted and washed 1X in EMEM media. Next, the cells were reconstituted in media and passed through a 35µm nylon mesh cell-strainer to remove cell clumping, and then added to the coated wells and incubated at 37°C for 15 minutes. This time was chosen based on binding rates of Neuro2A cells on both matrices, where binding was linear between 10 to 30 minutes (data not shown). At 15 minutes, the cells were removed, and the wells were washed 1X with PBS. Laminin- and fibronectin binding was determined using a cell counting kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, MO). This assay determines the

number of cells based on reduction of a tetrazolium salt into a yellow-dye, formazan by live cells and allows for detection based on absorbance at 450nm. Here, a solution containing CCK-8 was added to each well and incubated at 37°C for 1 hour. The mixture was then removed from the wells and absorbance was measured at 450nm.

SNA lectin blot—Lysate (2.5 μg) was separated on a 4-20% gel and then transferred to PVDF. Blotting was performed using biotinylated Sambucus nigra agglutinin, SNA, (Vector Labs, Burlingame, CA) followed by detection using an anti-biotin secondary antibody conjugated with HRP. Enhanced chemiluminescence substrate was then added to the membrane, and visualization was performed using X-ray film.

RESULTS

Isolation of Neuro2a cells with Tet-inducible expression of St8Sia2 or St8Sia4

In order to be able to vary levels of St8Sia2 or 4 transcripts in a controlled manner, the tet-ON inducible expression system was employed. The Addgene pCW57.1 plasmid was chosen since it obviated the need to perform two separate infections and selections by encoding both the tet-promoter and reverse tetracycline-controlled transactivator on a single plasmid. Viral particles were made using the pCW57.1 plasmids that contained either St8Sia2 or 4 sequences, which were then used to separately infect cells. Neuro2a cells were chosen based on studies that showed they expressed non-polysialylated NCAM but not St8Sia2 or St8Sia4 (30). These results were confirmed by qRT-PCR analysis of NCAM, St8Sia2, and St8Sia4 transcripts as well as Western blot analysis of lysates using anti-NCAM antibody (data not shown) and anti-polySia antibody (mAb735) (**Fig. 2.1A**), which recognizes polySia chains that contain >10 sialic acids (40,41).

Viral particles encoding St8Sia2 or St8Sia4 were used to separately infect Neuro2a populations, which were then selected for plasmid expression using puromycin. Each infection was then repeated, giving a total of four selected populations of cells, each of which was then compared to non-infected cells, N2A. Lysates from each population without DOX addition were subjected to SDS-PAGE using 4-20% gradient gels, followed by Western blotting with mAb 735 (**Fig. 2.1A**). In addition, blotting using an anti-β-actin antibody was performed as a sample loading control. Because there was some expression of polySia in all the non-induced populations, FACS and single clone selection were required to identify cells with negligible non-induced polyST expression.

FACS was performed with mAb 735 antibody and Alexafluor 488-labeled secondary antibody, followed by single cell clone selection. Clones were chosen based on lowest antibody binding to uninduced lysate when compared to control Neuro2a cells. Over 500 cells were sorted and the lysates of 30 randomly chosen clones were screened with and without 1 μg/mL DOX induction for 72 hours for mAb 735 antibody binding. Results were quantified by densitometry analysis. A single clone for each polySia that showed lowest levels of non-induced expression and significant levels of induced expression was chosen (**Fig. 2.1B**). These St8Sia2 and 4 clones will be referred to as ST2D7 and ST4F6, respectively.

PolySia and PolyST transcript expression increase as doxycycline (DOX) levels are increased

The levels of polySia synthesized by each clone as a function of increased DOX induction were measured by subjecting each to SDS-PAGE, followed by Western blotting

with mAb735. Specifically, concentrations of DOX that ranged from 1 ng/mL to 10 µg/mL were added to cells that were then grown for 24 hours. Cell lysates from ST2D7 (**Fig. 2.2A**) or ST4F6 (**Fig. 2.2B**) were then analyzed by Western blotting using both mAb735 and control anti-actin antibodies. Densitometry was performed and the ratio of polySia:β-actin calculated to control for loading variation (**Fig. 2.2C**). After 24 hours of DOX induction, the lowest concentration of DOX needed to detect polySia production in both clones was 10 ng/mL DOX. There was an approximately 50-fold increase in the polySia:β-actin ratio between 10 ng/mL and 10 µg/mL induction for 24 hr. for both clones. Moreover, the increases in relative expression levels of each enzyme's transcripts, normalized to RPL4 expression, roughly coincided with increases in polySia levels (**Fig. 2.2D**). An increase in polyST transcript levels of around 30-fold was seen for both ST2D7 and ST4F6 cells when uninduced cells were compared to those with 10 µg/mL DOX induction.

PolySia expression increases as a function of time of induction

Using a constant concentration of 1 µg/mL DOX induction, a time course analysis of polySia synthesis was performed. ST2D7 (**Fig. 2.3A**) and ST4F6 (**Fig. 2.3B**) lysates were sampled every 24 hours, subjected to SDS-PAGE and Western blotting, and densitometry was then performed to quantify mAb 735 and control anti-actin antibody binding. PolySia production for each polyST clone was calculated by first determining the polySia:actin ratios of antibody binding, then normalizing them to the value of the ratio at 96 hours with DOX induction set at 100% (**Fig. 2.3C**). Both polyST showed an increase in polySia expression as a function of time (**Fig. 2.3D**). Next, to determine the upper limits of polySia production, a similar time course of polySia production at the highest level of

DOX (10 μg/mL) induction was performed. At each time point, the levels of polySia product was roughly the same between the two clones (**Fig. 2.4**), suggesting that when using 10 μg/mL to induce expression, the limit of polySia expression is reached after 24 hours of induction.

Testing the relationship between increased polyST transcript levels and increased NCAM polySia glycoforms

The migration pattern visualized by anti-polySia antibody binding on Western blots after SDS-PAGE can give important comparative information regarding changes in polySia expressed on NCAM (28,42). The results in **Fig. 2.3A** and **B** show a shift of staining pattern as time of DOX induction and levels of polySia increase. The question arose, however, whether this change in migration was due to inherent polySia length differences, or if simply different levels of polySia present in samples could influence their migration pattern. To address this question and increase the resolution of NCAM glycoforms, gel separation was increased by using a 7.5% polyacrylamide gel instead of 4-20% gradient gel, and the electrophoresis was conducted at a low voltage for 18 hours**.** In addition, the amount of lysate loaded in each lane was normalized to the anti-polySia antibody binding levels derived from experiments similar to those shown in **Fig. 2.3A** and **B**. This technique was used by Seidenfaden et al. to analyze relative chain length of polySia expressed on NCAM (28). This normalization minimizes the possibility that changes in migration patterns are dependent on different levels of polySia in each sample (**Fig 2.5**). The results shown in **Fig. 2.6** show a striking similarity of polySia-NCAM banding patterns produced by St8Sia2 in ST2D7 cells between 24 and 96 hours. Likewise, little difference

in polySia-NCAM banding patterns was observed for St8Sia4 in ST4F6 cells from 24 to 96 hours of induction. These data show that, within the ranges of polySia production observed in our experiments, an over 3-fold increase in either polyST transcript levels and polySia production from 24 to 96 hours have negligible effect on migration patterns and, therefore, chain lengths, of polySia-NCAM.

Differences in polySia-NCAM glycoforms produced by St8Sia2 and St8Sia4

The results in **Fig. 2.6** also show that, from 24 to 96 hours of induction, at each time point St8Sia4 expression in ST4F6 cells resulted in a population of NCAM glycoforms that clearly migrated more slowly than those resulting from St8Sia2 expression in ST2D7 cells, denoted by the differences in arrowhead positions in **Fig. 2.6**. This difference in the trailing edge of polySia-NCAM resulting from St8Sia4 expression represents about 15% of the total amount of staining in each lane determined by densitometry. Remarkably, the relative amounts of these trailing species are similar from 24 to 96 hours of induction of the St8Sia4, during which enzyme transcript and polySia production increased significantly. In vitro studies comparing the chain lengths of the two enzymes using released radio-labeled polySia separated by strong anion exchange HPLC as well as anti-polySia antibody Western blot analysis, have also drawn the conclusion that St8Sia4 creates longer polySia chains, consistent with these results (28-30).

PolySia expression modulates cell adherence to extracellular matrix proteins

Cell-adhesion assays were performed using wells coated with fibronectin (**Fig. 2.7A**) or laminin (**Fig. 2.7B**), and the number of cells that bound to the matrices was

determined using an enzymatic assay. ST2D7 and ST4F6 cells showed equivalent amounts of polySia expression as a function of DOX concentration (**Fig. 2.3A,C**). For binding to fibronectin, both ST2D7 and ST4F6 showed a similar decrease in cell adherence as polySia expression increased (**Fig. 2.7A**). Specifically, both polysialyltransferases showed that 1µg/mL DOX induction led to a 15% decrease in binding compared to non-induced cells, while 10µg/mL showed around a 25% decrease in binding compared to non-induced cells. By contrast, expression of the two polySTs resulted in a difference in laminin-binding after DOX induction of 1µg/mL or 10µg/mL (**Fig. 2.7B**). After 1µg/mL of DOX induction, ST2D7 cells showed a 10% greater decrease in cell adherence compared to ST4F6 cells, $p<0.05$. This trend was also seen after 10µg/mL of DOX induction, where ST2D7 cells led to 13% greater decrease in adherence than observed with ST4F6 cells, $p<0.001$. Both of these differences in laminin adherence were repeated in three separate experiments and determined to be statistically significant. These results show that increased levels of polySia expression lead to a decrease in cell binding with similar inhibition observed in fibronectin adhesion, but not in inhibition to laminin.

DISCUSSION

The addition of polySia onto NCAM has been a well-studied process; however, how the polySTs regulate the complexity and size of the chains added to NCAM still remains poorly understood. NCAM is a member of the immunoglobulin super family, having five Ig domains (20). Polysialylation normally occurs on only two of the six N-linked glycans found in NCAM, both of which are in the Ig5 domain. These two N-linked sites are the most distal from the N-terminus and are referred to as the 5^{th} and 6^{th}

sites (31,43-45). Data from in vitro studies comparing polysialylation of point mutants of these N-linked sites on NCAM with wild-type NCAM, as well as in vivo experiments analyzing glycopeptides from polysialylated NCAM suggest the 6th site is the preferential site for polySia addition for both of the polySTs (31,43,44). Some polysialylation can be found on the 5th site, but not on the remainder of the four sites containing N-linked glycans.

While the N-linked site(s) of polySia addition is relatively constant on NCAM, the amount and chain length of polySia added onto NCAM have been shown to vary. One example is during chick brain development where chain length was measured using 1,2-diamino-4,5-methylenedioxybenzene (DMB) derivatization and separation using strong anion-exchange (SAX) chromatography. DMB specifically labels sialic acid at the α-keto group of carbon 1. After charge separation, labeled polySia chains can be detected by fluorescence. The amount of longer chain length polySia peaked between embryonic days 12 to 15, compared to earlier and later developmental times (46). Measurement of specific activity of the enzymes that synthesized the polySia at these developmental time points also showed an increase of over 4-fold (47). These activity measurements did not distinguish between the two polysialyltransferases, St8Sia2 and 4, but differences in chain length was associated with increased total enzymatic activity.

To delineate the difference between St8Sia2 and 4 effects on chain length, studies were performed using both knockout mice and overexpression of the polySTs in cell culture (30-32). In the first study, St8Sia2 null mouse brains contained half the amount of chains from polySia-NCAM that consisted of 21-35 monomers and only a third the number of chains longer than 35 monomers compared to St8Sia4 null mouse brains. In

addition, St8Sia2 knockout mouse brains synthesized ~40% less total polySia than St8Sia4 knockouts (32). A similar study using knockout mouse brains documented that the presence of chains around 90 sialic acid monomers was twice as abundant in the St8Sia4 deficient mice than the St8Sia2 deficient mice (31), although these differences could be caused by changes in the number and type of polySia producing cells, which are known to change when the polysialyltransferases are deleted (48-50). To simplify this problem, Kojima et al. created two stable Neuro2a cell lines that overexpressed either St8Sia2 or St8Sia4. Using these stable lines, polySia production and chain length were measured by radiolabeling the cells with [^{3}H] glucosamine followed by immunoprecipitating polySia from lysates and N-glycan release with PNGase F. St8Sia4 synthesized radiolabeled chains that eluted at higher salt concentrations from a SAX column than St8Sia2 chains, suggesting longer chains being produced by St8Sia4. It was also noticed that St8Sia4 incorporated around 30% more total radioactivity than the St8Sia2 cell line after immunoprecipitation, which would imply that St8Sia4 synthesized more polySia (30). Thus the results from metabolic radiolabeling and SAX separation of polySia from these cultured cells led to conclusions that differ than those from DMB labeling and SAX separation of polyST produced in St8Sia2 and 4 null mouse brains.

Since it is difficult to measure the activity of the polySTs, changes in transcript levels of the enzymes have been studied to determine their relationship to chain length. RT-PCR analysis of St8Sia2 or St8Sia4 mRNA and separation of polySia-NCAM by SDS-PAGE and Western blotting with anti-polySia were performed on several cell lines that expressed polySia-NCAM. Cell lines included in this study ranged from neuroblastoma, rhabdomyosarcoma, and acute myeloid leukemia cell lines, which varied

up to 8-fold in St8Sia4 transcript levels, but only 2-fold in St8Sia2 transcript levels. The cells differed in which polyST, or both, were expressed. A comparison of relative transcript expression of the polyST correlated with the results from the analysis of polysiaylated NCAM size (28). This result suggests an association of increases in transcript levels and decreased migration of polySia-NCAM. Because each cell type displayed differences in relative expression of St8Sia2 and 4, a means to modulate the expression of the individual polyST in the same cell would be very useful to compare differences between their expression levels and differences in NCAM polysialylation.

To further understand how St8Sia2 or 4 transcript levels affect polySia production, we generated a tet-inducible system that expressed either St8Sia2 or 4 in the same cell line, Neuro2a. This system allowed only one polyST transcript level to be modulated while other variables were held constant. Doxycycline dose response (**Fig. 2.2**) and time course assays (**Fig. 2.3**), followed by anti-polySia antibody Western blotting or qRT-PCR analysis, were performed. Results from these experiments showed that as polyST transcript levels increased, so did levels of anti-polySia binding up to 50-fold when comparing DOX induction of 10 ng/mL to 10 µg/mL for St8Sia2 and St8Sia4 after 24 hours. In this system, there appeared to be a maximum limit of polySia production for both polyST, reached after 24 hours of induction with 10 µg/mL of DOX (**Fig. 2.4**). This limit could be due to a lack of terminal α2,3 or α2,6 sialic acid on NCAM that is needed for polySia transfer. A previous study had showed that increased addition of α2,6 sialic acid in two neuroblastoma cell lines led to an increase in polySia production (51). It is also possible that CMP-sialic acid, the substrate for the polyST, could be limiting. A lectin blot after SDS-PAGE using *Sambucus nigra* agglutinin,

which binds α2,6 linked sialic acid, was performed using lysate from the 1 μg/mL DOX time course and uninduced cells to see if increased polyST expression might cause reduction in α2,6 sialylation, but no differences were seen between the samples for both polyST clones (**Fig 2.8**).

Differences in polySia-NCAM migration on SDS-PAGE gels was next analyzed using an anti-polySia Western blotting technique that used 7.5% acrylamide gel and long run times to maximize separation of NCAM glycoforms (28,42). Based on these data, increases in polyST transcript levels showed no significant difference in anti-polySia antibody binding patterns for either polyST (**Fig. 2.6**) from 24 to 96 hours of induction with 1 μg/ml of DOX. This result differs somewhat from a study that showed as polyST transcript levels increased so did the trailing edge of the anti-polySia antibody binding pattern (28). Based on our data, polyST transcript levels correlate with total polySia production. Unexpectedly, however, transcript levels had no significant effect on the binding pattern of anti-polySia antibody for each respective polyST expressing cell line. For St8Sia2 and St8Sia4, even though the amounts of polySia increased, the pattern of polySia-NCAM changed little. Because there is little difference in binding pattern, this suggests that the polysialylated NCAM glycoforms synthesized by 24 hours are similar to the polysialylated NCAM glycoforms synthesized after 96 hours.

Examining the polySia binding patterns in **Fig. 2.6**, there is a region of antibody staining for the separated St8Sia4-expressing cell lysates at each time of induction, consistent with slower migration due to either longer polySia chains or possibly more initiated chains, which is absent from the St8Sia2 cell lysates. This result is consistent with other cell-based and in vitro studies, that suggested St8Sia4 synthesized longer or

more polySia chains (28,30,44). This region corresponds to about 15% of the total anti-polySia staining on the blot for St8Sia4 cells and did not appear in St8Sia2 cells even after 96 hours of induction. Since loading was normalized to anti-polySia levels obtained from **Fig. 2.3**, the presence of the slower migrating NCAM glycoforms was not due to different levels of polySia loaded into the gel lanes, which can lead to misinterpretation of size distribution (**Fig. 2.5**). This result suggests that the products of two enzymes differ significantly in their migration pattern based on the sized distribution of the polySia they synthesize. For future studies, mass spectrometry analysis of the polysialylated N-glycans excised from the slowest migrating region from St8Sia4 gels could be compared to the polysialylated N-glycans from the faster migrating regions, along with corresponding regions of polySia-NCAM produced by St8Sia2. This comparison could likely determine the molecular cause of the slower migrating species produced by the St8Sia4 cells.

Currently, polySia's function in cell-matrix adherence has been characterized only by comparing endoneuraminidase (EndoN)-treated or untreated cells and then measuring binding to different matrices (21,39). The results of these studies were that the presence of polySia decreased adherence to laminin, fibronectin, cadherin, and poly-L-lysine 2- to 4-fold when compared to EndoN-treated cells. A study utilizing inducible expression of another glycosyltransferase, N-acetylglucosaminyltransferase V (GnT-V), showed that as GnT-V expression was increased, a significant decrease in fibronectin adherence was observed (52). It was interesting, therefore, to determine if polySia expression on NCAM caused a decrease in cell-matrix adhesion in a gradual manner similar to that observed with GnT-V and fibronectin. Experiments shown in **Fig. 2.7** demonstrate that the amount

of polySia produced by induction of both St8Sia2 and St8Sia4 gradually and directly decreased cell adherence to fibronectin and laminin (**Fig. 2.7**).

Comparison of adhesion of the two polyST-expressing cells on laminin (**Fig. 2.7B**) showed a significant difference while adhesion to fibronectin did not. Induction of St8Sia2 expression using 1µg/mL or 10µg/mL DOX led to a greater decrease in cell adhesion compared to the St8Sia4 expressing cells. It should be noted that this difference in inhibition of laminin binding is opposite to what might have been expected based on the results of the SDS-PAGE/Western blot assay (**Fig. 2.6**), which showed that a population of NCAM polySia glycoforms expressed by St8Sia4 migrated more slowly than those expressed by St8Sia2. This technique obviously cannot distinguish NCAM with longer polySia chains from NCAM that contains more polySia chains due to, for example, expression of polySia on additional, more highly branched N-linked glycans. As discussed above, experiments to identify the structural differences between the slower migrating population of polysialylated NCAM glycoforms and the remainder should be possible by eluting them from the gel and performing detailed glycomic analyses. Differences between the two polyST enzymes appear to result in both the synthesis of a small population of NCAM glycoforms with altered migratory behavior on SDS-PAGE, as well as a small, but significant, difference between the cells expressing each polyST and their adhesion to laminin. Both of these results suggest that the polySTs are not equivalent when comparing the addition and function of polySia synthesized.

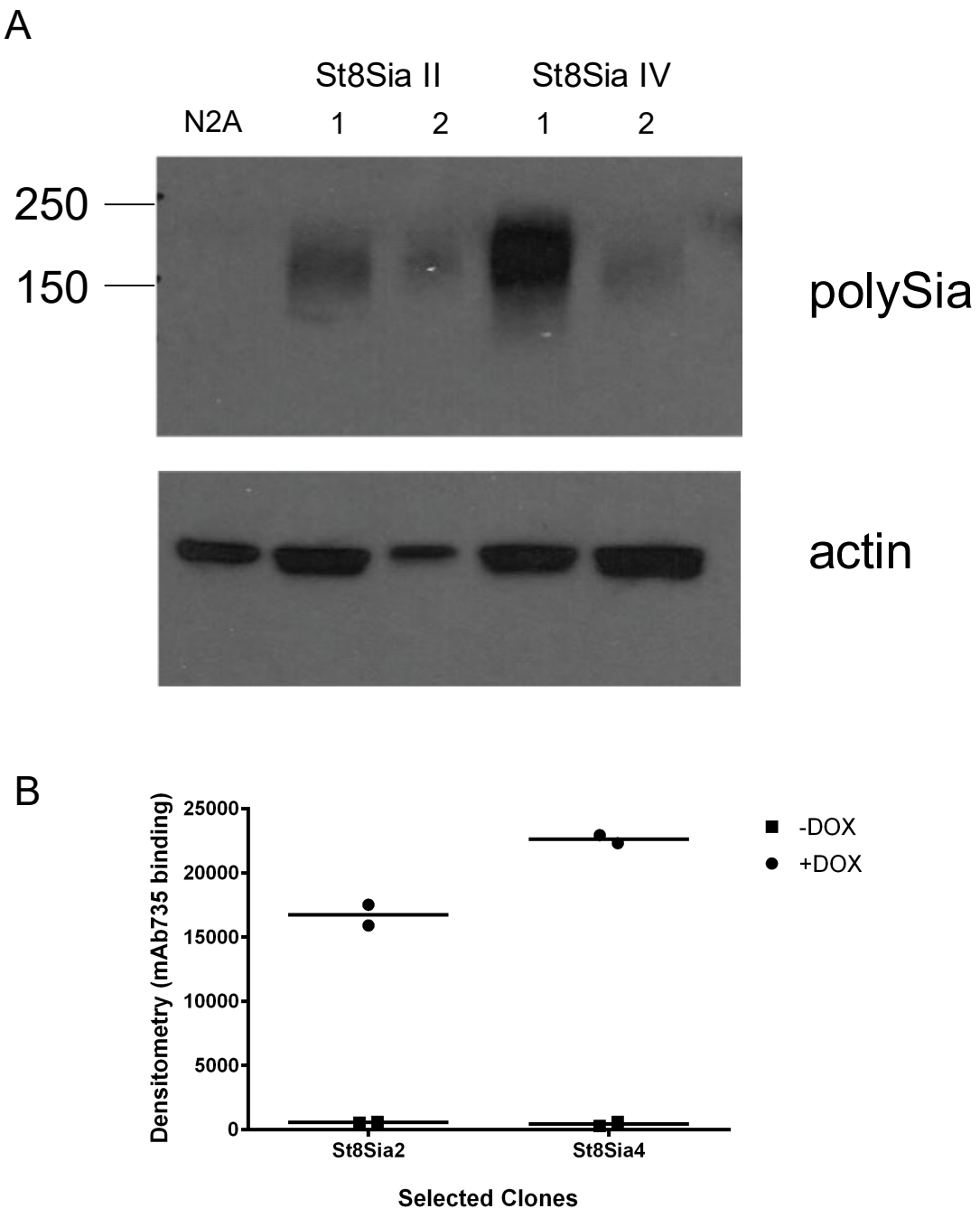

Figure 2.1. Selection of Neuro2A cells for Tet-inducible expression of St8Sia2 or St8Sia4. A, expression of polySia by cell populations infected with retroviral particles encoding tet-inducible expression of St8Sia2 or St8Sia4, selected by puromycin. This process of infecting and puromycin selection was performed twice for both polyST. Ten

μg of lysate of each selected population and uninfected Neuro2a cells (N2A) was separated on SDS-PAGE and Western blotted using the anti-polySia antibody (mAb735), followed by densitometric measurement. B, expression of polySia by single cell clones expressing St8Sia2 (ST2D7) or St8Sia4 (ST4F6) in the presence (▪) or absence of DOX (●) by dot blot analysis using the anti-polySia antibody (mAb735), n=2.

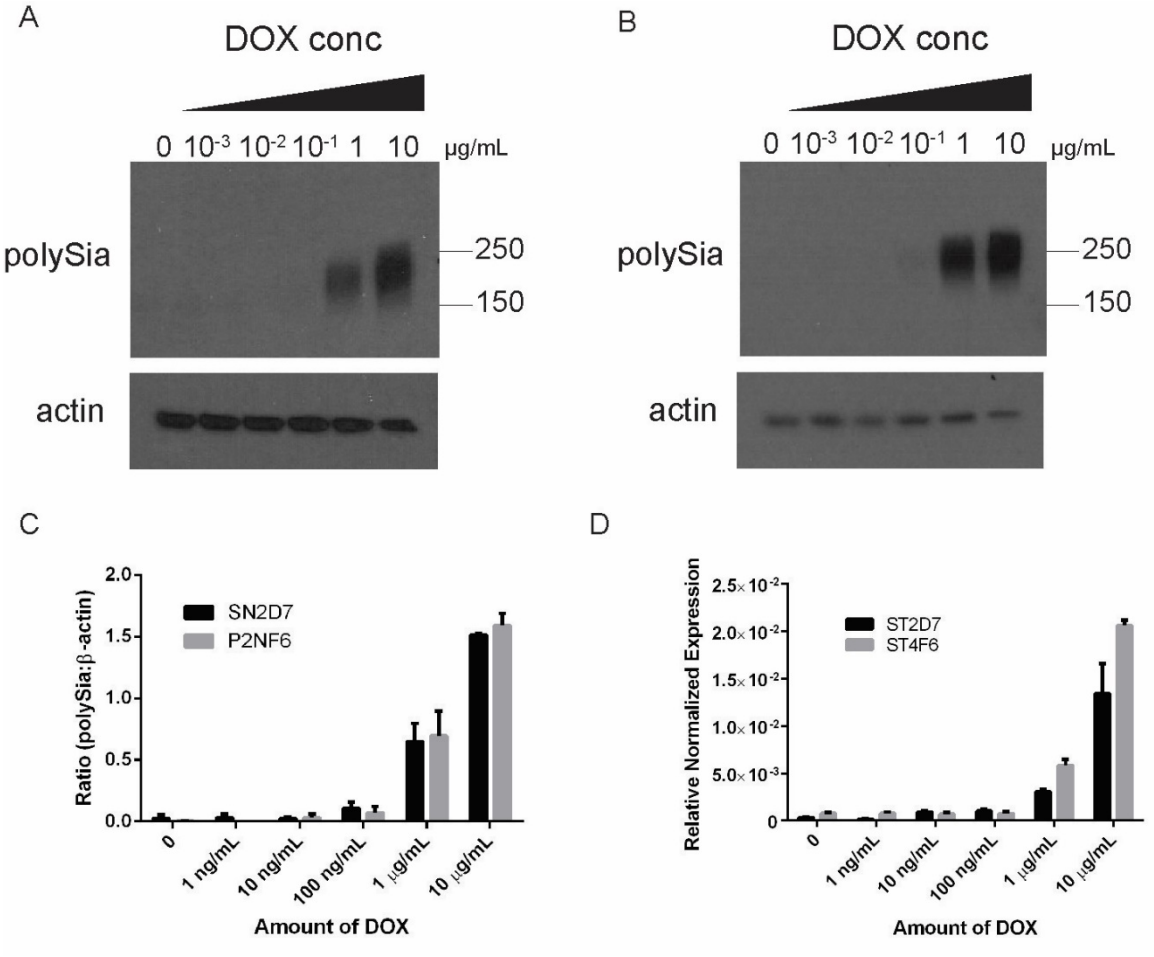

Figure 2.2. Induction of ST2D7 and ST4F6 cells as a function of doxycycline concentration. A, ST2D7 polySia expression by ST2D7 after incubation for 24 hr. in various concentrations of DOX, measured after SDS-PAGE, Western blotting with mAb735 (upper panel) or anti-beta actin antibody (lower panel); B, ST4F6, polySia expression after incubation for 24 hr. in various concentrations of DOX, measured after SDS-PAGE, Western blotting with mAb735 (upper panel) or anti-beta actin antibody (lower panel) ST2D7 and ST4F6 values are represented by black and gray bars, respectively; C, polySia expression as a function of various concentrations of DOX,

expressed as ratios of mAb735 staining/anti-beta actin antibody staining; Data are expressed as mean ± SEM; n=5. D, polyST relative transcript expression by qRT-PCR relative to RPL4 transcripts as a function of DOX concentration. Data are expressed as mean ± SEM; n=3. ST2D7 and ST4F6 values are represented by black and gray bars, respectively.

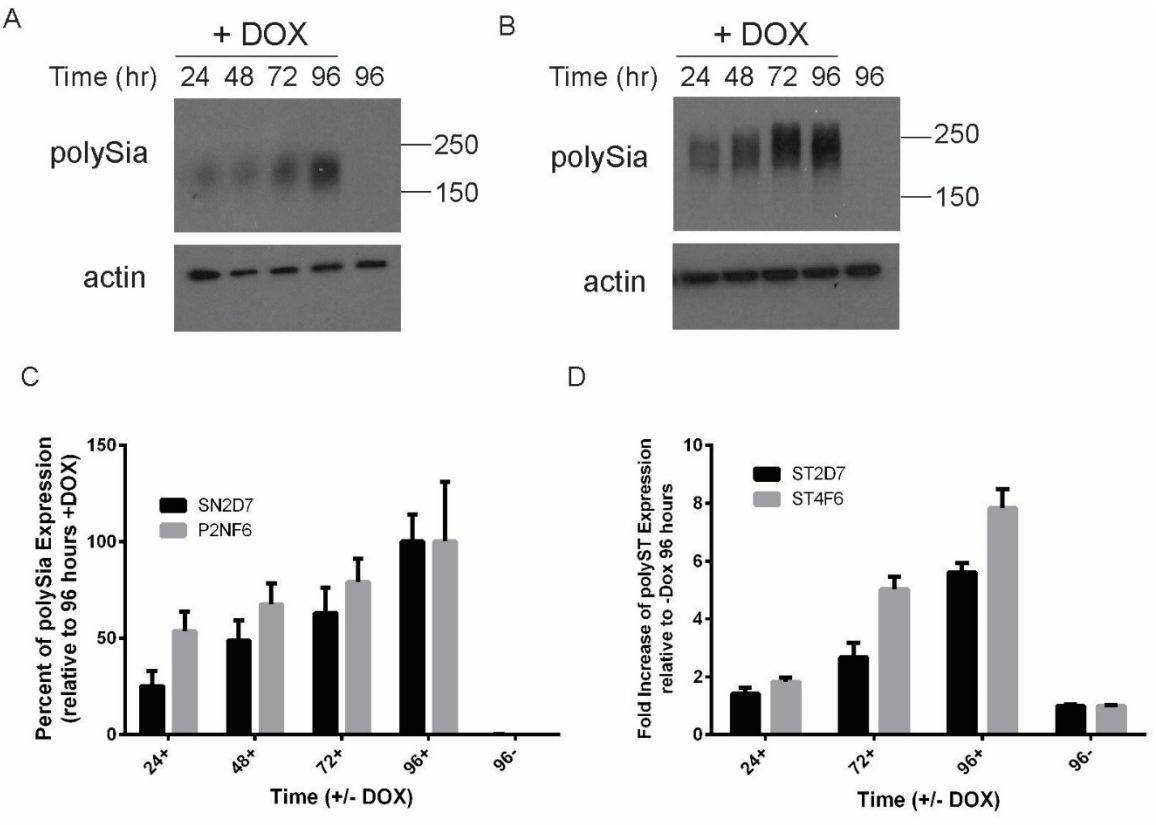

Figure 2.3. Time course of 1 µg/mL doxycycline induction for each polyST clone. A and B, ST2D7 (A) and ST4F6 (B) clones were induced with 1 µg/mL DOX and samples were taken every 24 hours for 96 hours. 10 µg lysate was blotted using mAb735 and anti-actin antibody. C, Densitometry was performed on blots and ratio of polySia:actin was normalized to 96 hours + DOX. Data are expressed as mean ± SEM; n=5. D, qRT-PCR for the respective polyST was done and expression denoted as fold increase compared to non-induced expression after 96 hours. Data are expressed as mean ± SEM; n=3. For both C and D, ST2D7 and ST4F6 are represented by the black and gray bars, respectively.

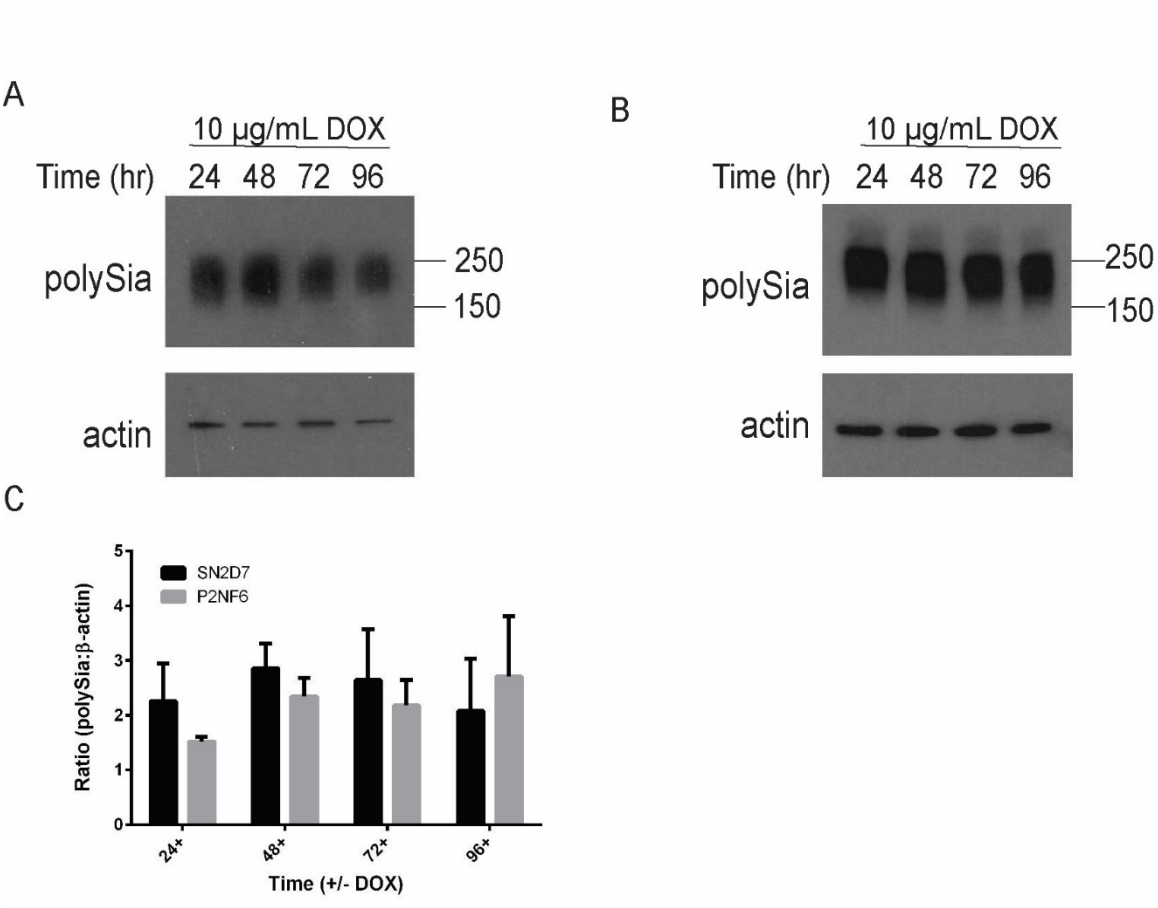

Figure 2.4. Time course of polySia expression after induction with 10 µg/mL doxycycline induction. A, polySia expression by ST2D7 cells after induction with 10 µg/mL DOX. Lysates were subjected to SDS-PAGE and blotted using mAb735 (top panel) and anti-actin antibody (bottom panel). B, polySia expression by ST4F6 cells after induction with 10 µg/mL DOX. C, Ratio of polySia to actin obtained from densitometry of blots shown in A and B. Data are expressed as mean ± SEM; n=3. ST2D7 (black bars) and ST4F6 (gray bars).

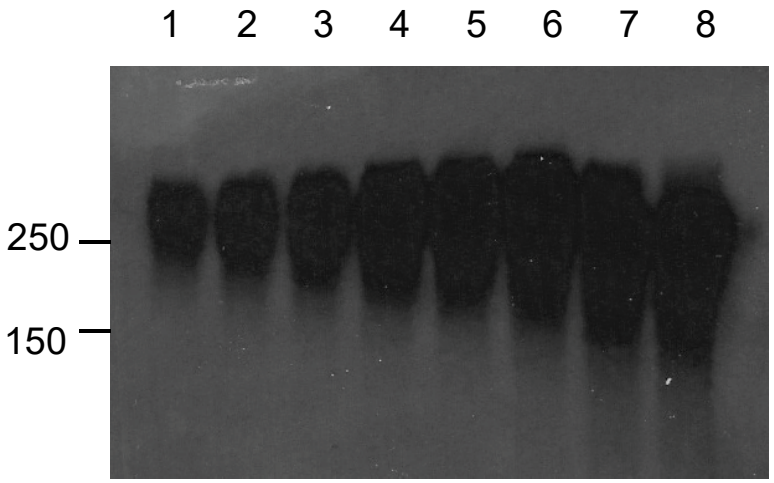

Figure 2.5. mAb735 Western blot analysis of varying protein amounts of same lysate. Varying amounts of lysate from ST4F6 that was induced for 24 hours with 10 µg/mL DOX were separated on 7.5% polyacrylamide and blotted with mAb 735. Each lane represents a different amount of protein. Lane 1, 0.313 µg; lane2, 0.625 µg; lane 3, 1.25 µg; lane 4, 2.5 µg; lane 5, 5.0 µg; lane 6, 10 µg; lane 7, 20 µg; lane 8, 40 µg.

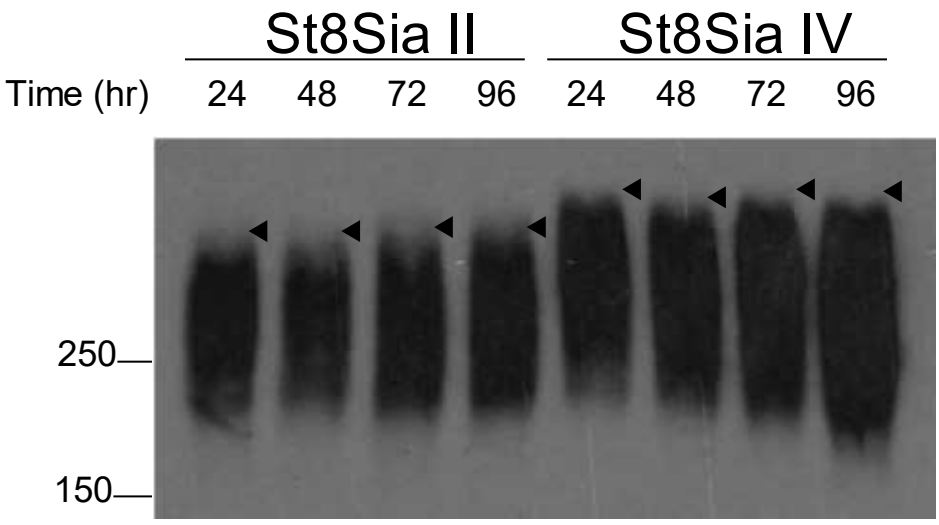

Figure 2.6. Western blot analysis of relative chain length using mAb735 blotting. Lysate from 1 μg/mL time course for ST2D7 and ST4F6 were separated on a 7.5% polyacrylamide gel at 30 Volts overnight and blotted using mAb 735. This technique allows increased separation of the sialylated forms of polySia-NCAM, where increased sialylation leads to slower migration. Lysate loaded was adjusted to have equal levels of polySia, determined by previous mAb735 blots (Fig. 3). Arrowheads denote the highest molecular weight of binding. (N=3)

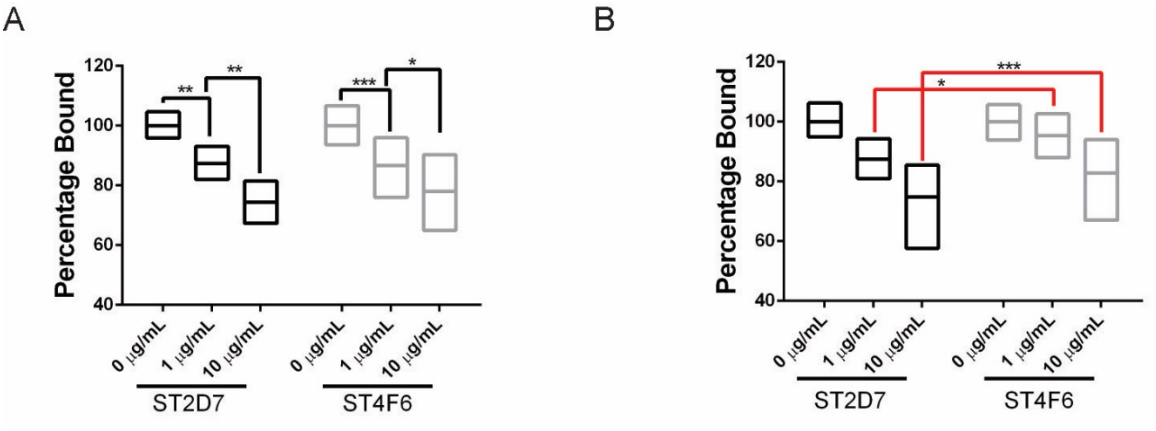

Figure 2.7. Increased polySia expression decreased cells adherence to fibronectin and laminin. PolyST expression was induced by 1µg/mL or 10µg/mL of DOX for ST2D7 and ST4F6 for 24 hours, followed by adding the cells (7.5 X 10^4 cells/well) into matrix coated wells for 15 min.; fibronectin, A; laminin, B. The number of cells adhered was determined following removal of non-adhered cells using the CCK-8 assay by measuring the absorbance at 450nm after 1 hour. CCK-8 absorbance values were compared to binding of the non-induced cells (0µg/mL) after 15 min., which was set at 100% binding. Values are n=7 or n=12 for fibronectin and laminin adhesion, respectively, for two to three independent experiments shown as a box-and-whisker plot. Significance levels are Student's T test indicated by * $p \leq 0.05$, ** $p \leq 0.01$, or *** $p \leq 0.001$.

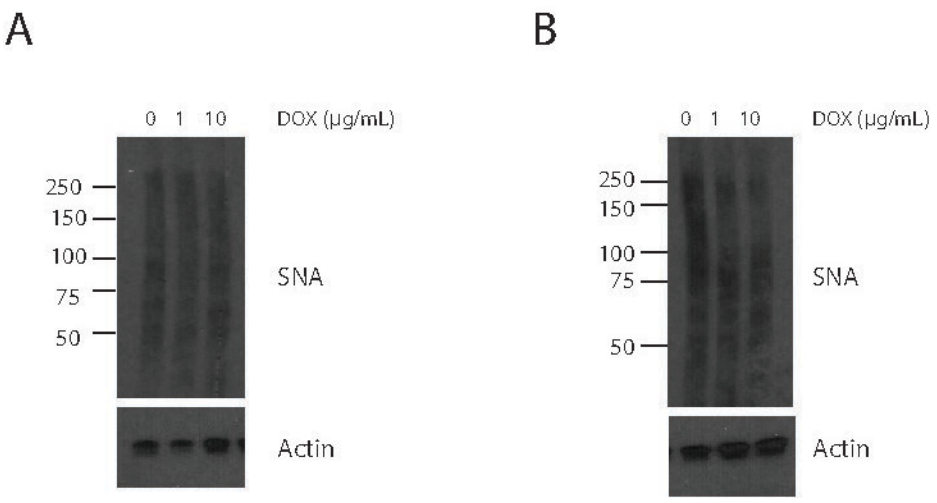

Figure 2.8. SNA lectin blot of ST2D7 or ST4F6 lysates induced by DOX. ST2D7 (A) or ST4F6 (B) cells were either not induced or induced by 1 or 10 µg/mL DOX for 24 hours. Protein lysates was then separated by SDS-PAGE on a 4-20% polyacrylamide gel. The gel was then transferred onto PVDF and blotted with biotinylated SNA lectin, which binds to α2,6 linked sialic acid, and detected using an anti-biotin secondary antibody that was HRP-conjugated.

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CHAPTER 3.

APPLICATIONS AND FUTURE PERSPECTIVES

To understand the relationship between polyST transcript expression and polySia biosynthesis and determine functional effects on cell adhesion, a tetracycline (tet)-inducible system was developed. The tet-inducible system for eukaryotes use was created in the early 1990s (1,2). It incorporated components from *E. coli* that respond to tetracycline addition along with a transcription activator from Herpes simplex virus that causes gene expression. The advantage of this system is it allows to modulate the expression of a particular gene based on the amount of tetracycline (or doxycycline, a tetracycline analog) that is added. This system comes in two forms, the first is a tet-off system where tetracycline-controlled transactivator (tTA) binds to the tet response element (TRE) and causes transcription to occur in the absence of tetracycline. Once tetracycline is added to the cells it is bound by the tTA and causes it to disassociate which decreases expression in a gradual manner depending on the amount of tetracycline added (1). The other tet system, tet-on, uses many of the same components as the tet-off system except the tTA is replaced with reverse tetracycline-controlled transactivator (rtTA). rtTA is a mutated version of tTA that does not associate with the TRE until doxycycline is added, which then initiates expression of the gene of interest in a similar dose-

dependent manner (2). Even though this system has been around for over 25 years, it has only been used in a handful of studies in the field of glycobiology (3-11). For this thesis work, this system was chosen since it provided a novel way to test how polyST transcript levels affected polySia production for both of the polySTs.

By using this system, new insights on the regulation of polySia biosynthesis were gained, in addition to validating previous polyST comparison studies. As expected, an increase in mRNA levels of the polySTs directly correlated with polySia production. To compare chain lengths synthesized as transcript and polySia production levels increased, a relative chain length assay was performed where separation of multiple NCAM glycoforms was done using a low percentage polyacrylamide gel along with an extended running time at low voltage. The amount of lysate loaded was adjusted for equal levels of polySia for each sample, which was determined from previous anti-polySia antibody Western blots. Interestingly, polySia chain length synthesized were not significantly affected by polyST expression levels for both polySTs within the range of polyST induction tested. This result disputes a finding in a previous study, that suggested that an increase in polyST expression—especially St8Sia4—leads to an increase in chain length (12). In addition, NCAM glycoforms expressed in the St8Sia2 and 4 clones appeared different. Specifically, the "trailing edge" for anti-polySia antibody binding was at a higher molecular weight in the NCAM glycoforms from the St8Sia4 clone compared to the St8Sia2 clone. Since negative charge would slow gel migration and sialic acid is negatively charged, this result could be explained by either St8Sia4 being able to

synthesize a small population of longer chain lengths or by initiating more polySia chains than St8Sia2. Lastly, in our system there was a limit of polySia production for St8Sia2 and 4 clones after 10 μg/mL of doxycycline was added to the cells for 24 hours. This was determined by the ratio of anti-polySia to anti-β-actin binding not significantly increasing over the next 96 hours of induction.

These results will need additional experiments to fully characterize how polySia biosynthesis is regulated. One of the first set of experiments would be to analyze the polySia glycopeptides and polySia N-glycans. To enrich for both polySia glycopeptides and N-glycans immunoprecipitations would be performed using an anti-polySia antibody (mAb 735). Mass spectrometry analysis would then be performed with these enriched fractions. These experiments could possibly explain why there was a difference between polySia production between St8Sia4 and II, and if polyST transcript levels affect where polySia addition occurs. Specifically, on NCAM there are six N-glycosylation sites, but only the final two sites can be polysialylated (5^{th} and 6^{th} sites) (13-15). Of these two sites, the 6^{th} site seems to be the preferential site for polySia addition for both polySTs (14,15). It would be of interest to determine if the relative levels of polysialylation at these two sites change as polyST transcript levels increases. Determining which N-glycan structures are polysialylated at low and high levels of polyST expression could give insight into possible preferential structures. These two set of experiments could determine if these polySTs have a preferred acceptor.

A more quantitative chain length assay than the SDS-PAGE/Western blotting

technique used in this thesis would allow for a better understanding of how polyST transcript levels affect chain length. As discussed in the Literature Review (Chapter 1), currently there is not a reproducible, reliable assay for measuring chain length. We have begun to explore creating a novel chain length assay that uses the selective exo-enzyme labeling (SEEL) technique (16). Here, St8Sia4 would add a modified CMP-Neu5Ac to the non-reducing end of each polySia chain. This modified CMP-Neu5Ac can have a fluorophore attached to the C5 of Neu5Ac. The SEEL reaction was successful using intact IMR-32 cells, as shown by a fluorescence band (Cy5 used) at 250 kDa after immunoprecipitation using an anti-polySia antibody (**Figure 3.1**). This same reaction was then performed with similar amounts of cells (5×10^7) followed by isolation of polySia N-glycans by PNGaseF treatment of the immunoprecipitated protein. These polySia N-glycans were then separated by reverse phase HPLC and detected for Cy5 fluorescence. Unfortunately, no signal for Cy5 was detected in the sample. More experiments are needed to optimize this technique, especially in the preparation of polySia N-glycans, but conceivable, this technique could be used to label after trypsinization polySia-expressing glycopeptides and determine their relative chain lengths.

In addition to demonstrating polyST transcript levels influence polySia biosynthesis, the tet-inducible system revealed differences in cell adherence when polySTs transcript levels were modulated. The first of these was that as polyST transcript expression increased the cell adherence decreased on matrices of laminin and

fibronectin. This result shows that polySia expression can modulate cell adherence in a dose-dependent manner. The other change seen was that St8Sia2 expression caused a statistically significant, greater decrease in cell adherence to laminin compared to St8Sia4 expression. Interestingly, this difference between the two polySTs was not seen with binding to fibronectin. The mechanism of how polySia could affect binding to these matrices is currently unknown, especially since NCAM has not been reported to directly bind to laminin or fibronectin (17). One possibility is that an increase in polySia causes a broad effect on cell surface protein interactions due to its highly anionic nature (13). This conclusion could explain the association between an increase in polySia expression and a decrease in cell adherence; however, it would not explain the differences seen between the binding results of St8Sia2 and 4 with laminin where St8Sia2 expression led to a greater decrease in laminin-binding compared to St8Sia4. To answer why there is a difference between the two polyST with laminin-binding, the binding partner(s) of laminin need to be determined. Analysis could be done by laminin-immunoprecipitations followed by proteomic analysis of binding proteins. To determine which of these binding partners to laminin is affected by polySia, laminin overlays would be performed. The binding of laminin would be done with or without colominic acid (bacterially produced polySia) addition, which could possibly replicate what occurs between NCAM-bound polySia during the laminin binding assays. These set of experiments will help to further characterize polySia's role in cell adherence.

Along with cell adherence assays, studies are planned to be performed to

investigate cell migration and cell-cell adherence. Cell migration will be studied by performing wound healing assays with fibronectin and laminin coated plates. In cancer, an increase in polySia is usually seen during metastasis (18) and is linked to the epithelial-to-mesenchymal transition, a step necessary for metastasis to occur (19). Based on this information, it is hypothesized that increased polySia production would result in enhanced cell migration. With cell-cell adherence, it has already been shown that polySia production would decrease aggregation based on homotypic repulsion of the two anionic chains on differing cells. Currently, it is unknown if this is a gradual process as shown with cell adherence to the matrices based on polySia production.

Another question that can be answered with these cells is why there is a limit of polySia production after the addition of 10 μg/mL of doxycycline for 24 hours. For this result there are two predictions: 1) a limit in CMP-Neu5Ac and 2) lack of α2,6 or α2,3 terminal sialic acids needed to prime the synthesis of polySia. Two recent studies characterized a hypermutation in UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine-kinase (GNE) which is involved with the biosynthetic pathway for CMP-Neu5Ac (20,21). This mutation was seen in patients that had sialuria, which is the overproduction of CMP-Neu5Ac causing it to be present in patients' urine. When this mutated form of GNE was expressed in Chinese hamster ovarian (CHO) cells or in a transgenic mouse an increase in polySia-NCAM was seen, although there was not an increase in α2,6 or α2,3 addition to glycoproteins as determined by lectin blots (20,21). This would suggest that polySia could be regulated by the amount of CMP-Neu5Ac

available for transfer. To test increased levels of CMP-Neu5Ac effect in our system, mutated GNE could be transfected into the inducible cells and add 10 μg/mL of doxycycline to the cells. The amount of polySia produced would be determined by Western blot analysis with the anti-polySia antibody. If an increase in polySia production occurs, as seen in the previous studies of this GNE mutation, it would suggest that the amount of CMP-Neu5Ac might be limiting in polySia production. Another possible limiting step is the amount of terminal sialic acids to which the polySia could be transferred. Georgopoulou et al. transfected a St6Gal from rat into neuroblastoma cell lines that already expressed polySia on NCAM. Transfection of the St6Gal led to an increase in polySia production, determined by Western blot for polySia (22). Both of these proposed experiments could possibly answer why an upper limit of polySia production is seen in our system.

Currently, experiments to study α2,3 and α2,6 sialic acid production in the polyST expressing cells are being and have been performed. The first set of experiments are transcriptome analysis of St6Gal (St6Gal I and II) and St3Gal (St3Gal IV and VI) expression for both the time course and dose response experiments to determine if an increase in polySia affects any of these genes being expressed. In addition to transcriptome analysis, a lectin blot using *Sambucus nigra* lectin, which binds α2,6-linked sialic acid, has already been performed on both polyST clones after doxycycline induction and little differences were seen between induced and non-induced cells. It would be interesting to knockdown expression of St6Gal or St3Gal family members in

our cells and test the effect on polySia production when adding varying amounts of doxycycline.

As discussed above, the cells developed as part of this dissertation are a powerful tool in the study of polySia biosynthesis and function. New observations have been presented during the characterization of the cells which require further analysis to obtain a better understanding on polySia production on NCAM. Characterizing the polysialylated NCAM glycoforms, separation using SDS-PAGE showed differences in NCAM glycoforms synthesized by the two polySTs, where St8Sia4 polysialylated NCAM glycoforms showed slower migration than St8Sia2 polysialylated NCAM glycoforms. Cell adhesion assays showed a significant reduction in cell adherence on laminin for St8Sia2 expressing cells compared to St8Sia4 expressing cells. These two results suggest that St8Sia2 and 4 show differences in both their biosynthetic properties, as well as functional differences in their polysialylated products.

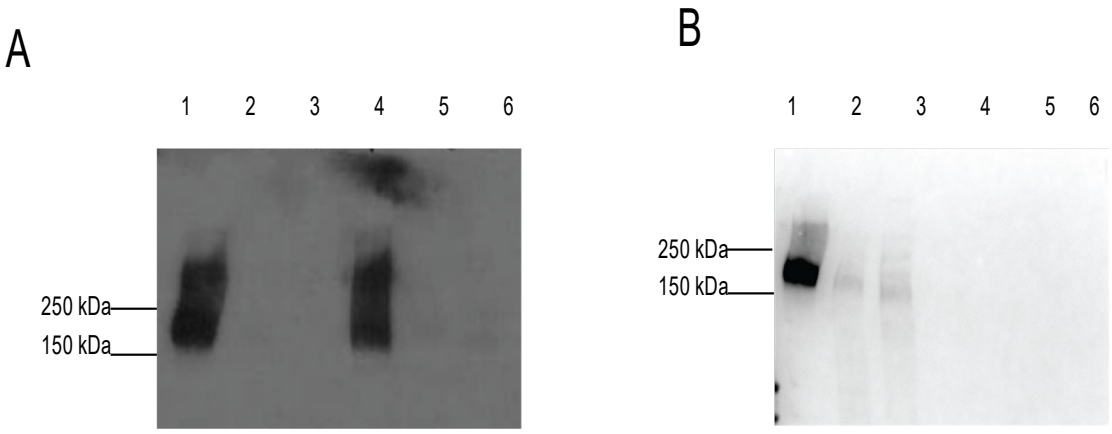

Figure 3.1. SEEL reaction of IMR-32 Cells using St8Sia4 and CMP-Neu5Ac modified with Cy5 fluorophore. Following SEEL reaction lysate was immunoprecipitated by an anti-polySia antibody then separated on SDS-PAGE and transferred onto PVDF membrane. The membrane was then either blotted using anti-polySia antibody (A) or Cy5 fluorescence was detected using a Bio-rad ChemiDoc Imager (B). Lanes 1-3 consist of SEEL reaction using CMP-Neu5Ac-Cy5 substrate while lanes 4-6 used unmodified CMP-Neu5Ac for SEEL reaction. Removal of anti-polySia bound protein was performed two ways, first using high pH buffer (lanes 1 and 4) followed by boiling off beads in Laemmli buffer (lanes 2 and 5), while lanes 3 and 6 were unbound fractions.

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