Identification of Polysialyltransferase Sequences Required for

Substrate Recognition and Polysialylation

BY

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THESIS

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This thesis is dedicated to all of the individuals in the past and present who have encouraged me, assisted me, worked with me, supported me mentally and emotionally through the years, and helped me solidify my passion for science and medicine. It is for their unwavering support that this thesis work was even possible.

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JLZ

PREFACE

The modification of the neural cell adhesion molecule (NCAM) with polysialic acid (polySia) has been found to be absolutely necessary for proper development of the mammalian nervous system, and interestingly has been found to occur in the advanced stages of several human tumors, where it promotes the ability of these tumors to spread to other parts of the body. Research has shown that the addition of polySia to substrates requires an interaction between the substrate and the polysialyltransferase enzymes that add polySia, but the precise biochemical mechanism remains unknown. Therefore, the goal of this thesis work is to determine how this interaction takes place in order to lay the foundation for the potential development of therapeutics to alleviate this process in human disease.

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LIST OF ABBREVIATIONS

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
CDG	Congenital disorder of glycosylation
CUB	Complement C1r/C1s, Uegf, Bmp1 domain
DAPI	4', 6-diamidino-2-phenyl-inidole, dihydrochloride
DMEM	Dulbecco's modified Eagle's medium
ER	Endoplasmic reticulum
F5/8	Coagulation factor 5/8 type domain
FBS	Fetal bovine serum
FN	Fibronectin type III repeat
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
Glc	Glucose
GlcA	Glucuronic acid
GlcNAc	N-acetylglucosamine
Glu	Glutamic acid
IdoA	Iduronic acid
Ig	Immunoglobulin-like domain
LSD	Lysosomal storage disorder
Lys	Lysine
M3	Motif III

MAM	Meprin/A5 protein/PTPmu domain
Man	Mannose
NCAM	Neural cell adhesion molecule
NRP-2	Neuropilin-2
PBR	Polybasic region
PBS	Phosphate buffered saline
PolySia	Polysialic acid
PolyST	Polysialyltransferase
PSTD	Polysialyltransferase domain
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
Sia	Sialic acid
SML	Large sialylmotif
SMS	Small sialylmotif
SynCAM 1	Synaptic cell adhesion molecule 1
Thr	Threonine
Xyl	Xylose
VSMS	Very small sialylmotif

SUMMARY

ST8Sia IV (PST) and ST8Sia II (STX) are α2, 8-sialyltransferases that catalyze the addition of long homopolymers of sialic acid residues (polysialic acid) on the glycans of a limited number of protein substrates. The neural cell adhesion molecule (NCAM) is the most abundant polysialylated protein, and polysialic acid (polySia) serves as an anti-adhesive glycan that prevents cell-cell interactions mediated by NCAM and other nearby adhesion molecules and modulates NCAM cell signaling. Previous studies have demonstrated that polySia is absolutely necessary for the proper development and plasticity of the mammalian nervous system, and plays important roles in memory formation, olfaction, and post-injury neuron repair in the adult. More recent studies have demonstrated that the polysialylation of synaptic cell adhesion molecule 1 (SynCAM 1) and neuropilin-2 (NRP-2) is critical for modulation of synapse formation and dendritic cell function and migration, respectively. Abnormal levels of polySia have been observed in several human diseases, including Alzheimer's disease, schizophrenia, and Huntington's disease. Notably, polySia is highly expressed in several different human tumors and is believed to promote their invasiveness and metastatic potential. Therefore, polySia is a versatile post-translational modification of select glycoproteins that serves both necessary and beneficial functions throughout mammalian life, but can also be detrimental if expressed aberrantly.

The demonstrated roles of polySia during development, in the adult, and in disease make the process of polysialylation a potential target for the development of therapeutics aimed at the alleviation of human disease, especially cancer. Despite our knowledge of the biological significance of polysialylation, the precise biochemical mechanism by which the polysialyltransferases (polySTs) recognize and polysialylate their substrates was poorly understood at the beginning of my thesis work. Understanding this mechanism is a prerequisite for the development of therapeutics. Early on, we developed an overarching hypothesis concerning the mechanism of polysialylation that has been supported by a variety of studies on NCAM polysialylation from our laboratory and others, that states that polysialylation is a protein-specific process that requires an initial polyST-substrate protein-protein recognition step. With this current working model in mind and an understanding of the roles that polySia plays, the overall goal of my thesis work was to identify specific sequences within the polySTs, PST and STX, that are responsible for the effective recognition and subsequent polysialylation of NCAM, SynCAM, and NRP-2.

Previous data suggested that residues prior to the catalytic region of PST are critical for NCAM polysialylation. In order to identify the PST residues required for substrate recognition, I focused on amino acids 1-140 of PST and created a series of PST proteins for competition studies in SW2 small cell lung carcinoma cells. I expected that overexpression of catalytically inactive proteins containing PST residues required for substrate recognition would compete with the endogenous PST in these cells and block polysialylation of endogenous NCAM. I created a full-length catalytically active soluble PST lacking its cytosolic and transmembrane regions (sPST), a full-length catalytically inactive PST (PST H331K), and PST molecules consisting of the first 140, 127, 71, and 62 residues (PST140, PST127, PST71, and PST62), as well as a soluble form of PST140 (sPST140). While the overexpression of PST and sPST increased polySia expression in SW2 cells, as expected, overexpression of PST H331K, PST140, sPST140 or PST127 led to an overall decrease in polySia expression, suggesting that these proteins are able to compete with endogenous PST for NCAM recognition. In contrast, SW2 cells overexpressing PST71 or PST62 exhibited no significant change in polySia expression suggesting that these proteins lack the substrate recognition sequences. This conclusion was further supported by co-immunoprecipitation studies that showed that PST71 and PST62 do not bind to NCAM as effectively as the other PST molecules that served as competitive inhibitors of NCAM polysialylation. Collectively, these results suggest that amino acid sequences within residues 71 and 127 of PST are critical for NCAM recognition and binding.

Next, I examined this PST region for specific residues that mediate NCAM recognition. I focused on Arg^{82} and Arg^{93}, two residues located in a polybasic region (PBR) previously shown by our laboratory to be critical for NCAM polysialylation but not overall enzyme activity. I found that a PST H331K protein containing an R82A, R93A, or R82A/R93A mutation could no longer effectively reduce SW2 cell NCAM polysialylation by endogenous PST. I was able to quantitatively determine that Arg^{82} and Arg^{93} were both required for the competitive effect of PST H331K utilizing a triple expression system in COS-1 cells in which NCAM, PST, and a PST H331K competitor were expressed in a 1:1:6 ratio. I found that the single alanine mutations reduced the ability of PST H331K to compete with wild-type PST and decrease NCAM polysialylation, whereas the double mutation eliminated the

ability of PST H331K to compete. These results showed that PST PBR basic residues Arg^{82} and Arg^{93} are critical for the recognition of NCAM.

To understand whether the recognition and polysialylation of other substrates also requires these PST PBR residues, and whether similar sequences in STX are required for substrate recognition, I expanded my studies to more thoroughly examine the PBR residues in both polySTs, and their role in the recognition and polysialylation of NCAM, SynCAM 1, and NRP-2. Each polyST PBR basic residue was mutated individually to alanine in the wild-type PST/STX or PST H331K/STX H346K background for polysialylation and competition studies, respectively. These studies revealed that the polyST requirements for polysialylation and recognition were unique for each polyST-substrate pairing. I found that PST Arg^{82} is critical for the polysialylation of all three substrates. Efficient NCAM polysialylation also required Arg^{93}, while NRP-2 polysialylation required Lys^{99}, and SynCAM 1 polysialylation required both Arg^{87} and Lys^{99}. A larger set of STX PBR residues are involved in NCAM recognition, including Arg^{97} (analogous to Arg^{82} in PST), Lys^{98}, Lys^{102}, and Lys^{108} (analogous to Arg^{93} in PST). In contrast, STX Lys^{98}, Lys^{102}, Lys^{108}, Lys^{114}, and Lys^{118} were all required for effective polysialylation of SynCAM 1 by STX, and interestingly, the contribution of each residue varied significantly. Analysis of NRP-2 polysialylation by STX PBR mutants was inconclusive. The competition studies demonstrated that the PBR residues identified as critical for substrate polysialylation were the same residues that are required for substrate recognition. I concluded that the polyST PBR is critical for the recognition of polyST substrates and that unique combinations of PBR basic residues are used for recognition in each polyST-substrate combination.

The results presented in this thesis work demonstrate that polysialylation is a highly protein-specific process, and identify specific residues in a polybasic region of each polyST that is responsible for mediating the recognition of substrates. Strikingly, the sets of PBR residues required for NCAM, SynCAM 1, and NRP-2 recognition overlap, and yet contain unique members, suggesting the opportunity for devising strategies to selectively block global polysialylation or the polysialylation of a single substrate. It is my hope that these results will ultimately contribute to the creation of potential inhibitors to target the polyST-substrate interaction and thereby serve as therapeutics to alleviate human disease.

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Protein glycosylation

The enzymatic addition of glycans onto the side chains of specific amino acid residues represents the most common and diverse post-translational modification of newly synthesized proteins. It is estimated that a majority of cellular proteins processed in the secretory pathway and destined for the cell membrane or secretion are glycosylated, as are some nuclear and cytosolic proteins. Glycan chains are synthesized on target proteins by approximately 250 enzymes known as glycosyltransferases, which catalyze the addition of a nucleotide-activated sugar residue onto an existing protein-attached glycan acceptor [1, 2]. Acting sequentially, glycosyltransferases can synthesize glycan chains on proteins that can be as simple as a single monosaccharide, or as complex as a large, branched polymer chain consisting of hundreds of sugar residues, utilizing only ten different monosaccharides: N-acetylglucosamine (GlcNAc), mannose (Man), glucose (Glc), galactose (Gal), N-acetylgalactosamine (GalNAc), fucose (Fuc), sialic acid (Sia), glucuronic acid (GlcA), iduronic acid (IdoA), and xylose (Xyl) [2-4]. With differential arrangement of these ten sugars into glycan chains by the glycosyltransferases, as well as further chain modification with phosphate, sulfate, acetate, and/or phosphorylcholine [1], the full diversity of mammalian glycans is created and is estimated to consist of over 7,000 total structures [2].

Addition of a glycan chain to a particular protein may alter that protein's proteolytic resistance, solubility, stability, local structure, and half-life [5, 6]. Protein glycosylation in mammals has also been well documented to contribute to, promote, or modulate a plethora of critical biological functions [reviewed in 7]. In the secretory pathway, glycans added to newly synthesized proteins in the endoplasmic reticulum influence protein folding by serving as ligands for the lectin chaperone proteins calreticulin and calnexin, thus contributing to ER quality control surveillance mechanisms ensuring the transit of only correctly folded glycoproteins forward to the Golgi apparatus [8-11]. At the cell surface, protein-attached glycans can modulate interactions with the extracellular environment, including cell-cell adhesion properties, cellular signaling, and macromolecule interactions [7, 12-16]. Furthermore,

cell surface glycans can unintentionally influence the attachment and invasion of pathogens. As examples, sialic acid residues that terminate a glycan chain can serve as a receptor for hemagglutinin proteins on the surface of a variety of viruses including influenza variants [17-21], and can also be utilized by a pathogen to block antigenicity by transferring these sialic acid residues to its own surface [22-24]. Furthermore, other studies have pointed to a variety of contributions of glycan structures in the structural integrity of tissues [25-26], immune surveillance [27], inflammation [28], autoimmunity [29-30], tumor metastasis [31], and the regulation of cytosolic and nuclear functions [32].

The process of glycosylation is divided into two major categories based on which amino acid the carbohydrate chain is attached to. These two categories are (1) N-linked glycosylation, in which a glycan chain is attached to an asparagine (Asn) residue in the Asn-X-Serine/Threonine consensus sequence (where "X" is any amino acid except proline), and (2) O-linked glycosylation, in which a glycan chain is attached to a serine (Ser) or threonine (Thr) residue without a consensus sequence. In addition to these forms of glycosylation, two others mechanisms are worth nothing. First, glycosylation can also occur on plasma membrane associated lipids in which a glycan chain is synthesized on the lipid's membrane-anchoring ceramide moiety [33]. These glycolipids can be modified simply by one or two sugars, or extensively by sets of Golgi glycosyltransferases [34]. Secondly, proteins can be anchored to the extracellular side of a cell's plasma membrane via a glycophosphatidylinositol (GPI) linkage, in which a protein's C-terminal is anchored to the membrane via an inositol phosphate group attached to a series of carbohydrate residues [35-36]. The two major forms of glycosylation, namely N- and O-linked forms, will be discussed in detail below.

N-linked glycosylation

Asparagine (N-linked) glycosylation of proteins is a highly extensive post-translational modification that results in the covalent attachment of a glycan chain via an amide linkage onto select asparagine residues in a polypeptide chain [37]. This process occurs within the secretory pathway and thus mainly affects proteins that are destined for secretion, the plasma membrane, endoplasmic reticulum (ER), Golgi apparatus, or endosomes. Unlike other protein post-translational modifications, N-linked glycosylation is unique in that it begins co-translationally

on polypeptides that are actively being synthesized in the rough ER. Furthermore, despite great diversity amongst the final glycan products, all N-linked glycans invariably will begin with a GlcNAc residue covalently attached to the asparagine side chain and have a common core structure of two GlcNAc and three Man residues [38]. Their diversity lies in the processing of the glycan's outer branches.

The production of N-linked glycans is the result of sequential chain processing by glycosyltransferases and glycosidases in the ER and the Golgi apparatus compartments [39] and is outlined in Figure 1. Synthesis begins independently of the nascent polypeptide on the cytosolic face of the ER membrane with the creation of a 14-sugar oligosaccharide precursor consisting of three Glc, nine Man, and two GlcNAc residues ($Glc_3Man_9GlcNAc_2$) attached to a lipid, dolichol diphosphate. The $Glc_3Man_9GlcNAc_2$ oligosaccharide is transferred by an oligosaccharyltransferase *en bloc* to an asparagine residue within the proper signal sequence on a nascent polypeptide [33, 40]. In the ER, these protein-attached glycans are trimmed of their terminal two Glc residues and interact with chaperones that are part of the ER quality control system until the attached protein is folded properly and the final Glc is removed. The properly folded protein exits the ER and moves to the cis-Golgi possessing $Man_9GlcNAc_2$ high mannose N-glycans. In some cases, these glycans pass through the secretory pathway unchanged. However, they are frequently modified extensively by a series of glycosidases and glycosyltransferases in the cis-, medial-, and trans-Golgi, leading to the creation of a diverse field of hybrid and complex N-glycans [1]. As an example, CHO cells have been shown to have complex N-glycans that can carry over 60 sugar residues [41].

Over the past forty years, the ability of cells to perform N-linked glycosylation has been well documented to be a critical process that is absolutely necessary for proper mammalian development and survival. One of the first studies to show the significance of N-glycans for proper embryonic development was performed by Surani, M.A.H. [42], who utilized tunicamycin to study the significance of mouse embryonic N-glycosylation. Tunicamycin is a GlcNAc analog and competitively inhibits the enzyme UDP-GlcNAc: dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT), preventing the synthesis of the dolichol lipid-linked precursor glycan ($Glc_3Man_9GlcNAc_2$) and thus inhibiting the formation of N-linked glycans. Upon treatment of isolated mouse embryos with tunicamycin, it was found that upon reaching the 32-cell stage, these cells remained uncompacted

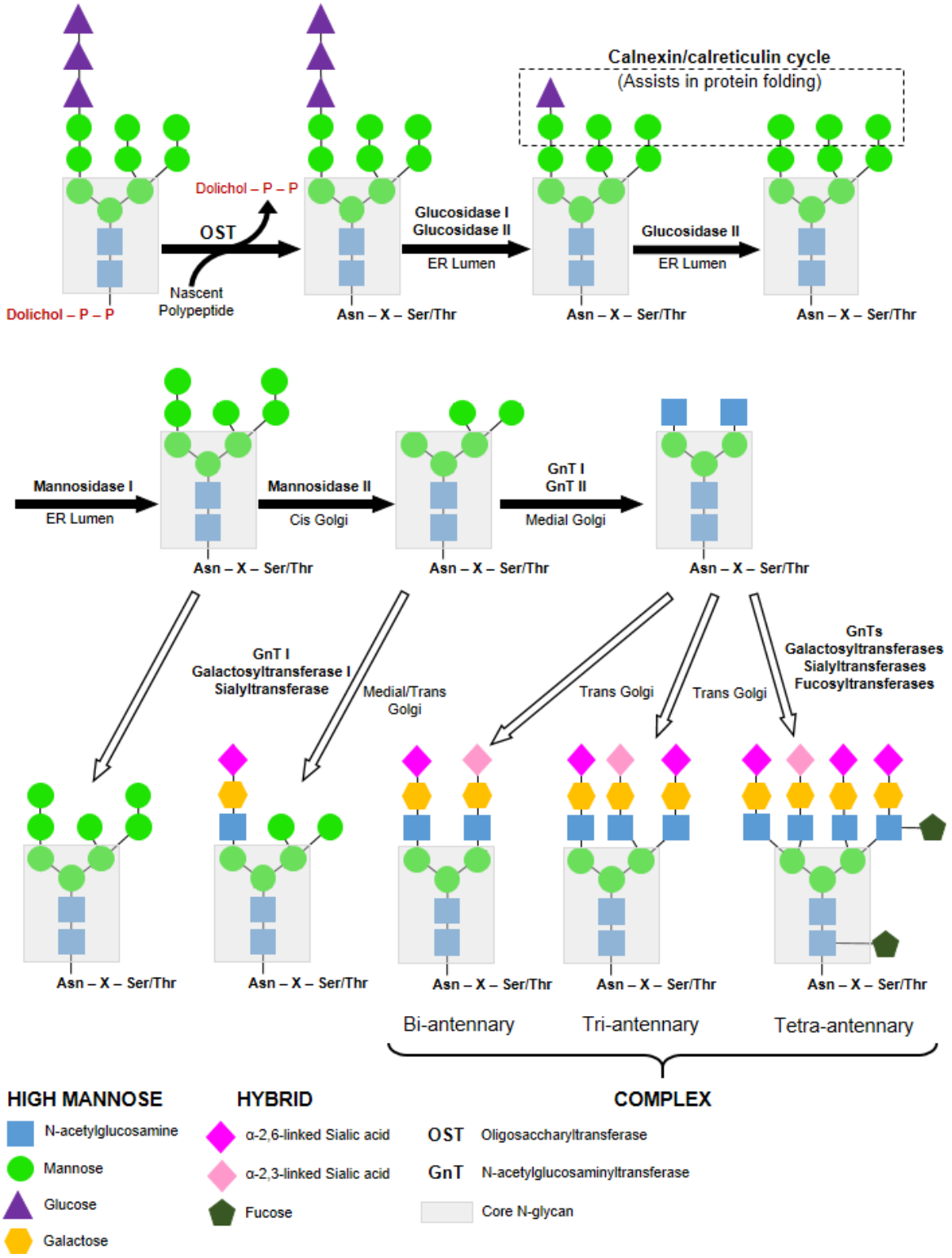

Figure 1. Overview of the N-linked glycosylation synthesis pathway. A $Glc_3Man_9GlcNAc_2$ precursor is transferred to a nascent polypeptide arginine *en bloc* in the ER, is trimmed of two Glc residues, then aids in protein folding. Upon protein passage to the Golgi, the N-glycan is acted upon by a series of glycosidases and glycosyltransferases, creating high mannose, hybrid, and complex N-glycans.

and were unable to form mature blastocysts, thus preventing the formation of a mature embryo and suggesting a lethal phenotype. This suggestion was solidified by Marek *et al.* [43], who constructed and characterized mice with a GPT gene deletion. It was found that mouse embryos that were homozygous recessive for the GPT-null phenotype demonstrated complete degeneration of all embryonic and extraembryonic cells as early as embryonic day 5.5, rendering these embryos unviable and indicating that N-glycans are absolutely necessary for cellular viability and organismal function during embryonic development. Further studies that allowed N-glycans to initially form, but prevent normal modifications in the Golgi, indicate that a full complement of complex N-glycans is critical for normal mammalian development. Knockout studies in which mice were created devoid of the *MgatI* gene that encodes GlcNAc-transferase-I (GnT-I), which allows for conversion of high mannose glycans to hybrid and complex N-glycans, were found to have improperly formed neural tubes and vascularization along with situs inversus of the heart, and these embryos did not survive past embryonic day 10 (mid-gestation) [44-46]. Furthermore, mice with a deficiency of α-mannosidase II survive embryonic development and look similar to wild-type littermates during adulthood, however, these mice develop a dyserythropoietic anemia accompanied by splenomegaly, with early death resulting from kidney failure [47-49].

O-linked glycosylation

In contrast to N-linked glycosylation, glycans can also be synthesized through glycosidic linkages (O-linked) to the side chains of serine or threonine residues within a polypeptide, as well as to hydroxylysine (collagen) or tyrosine (glycogenin). Unlike N-linked glycosylation, the process of O-linked glycosylation is completely posttranslational (no co-translational modifications), initiating in the ER for most glycoproteins, with further modifications occurring in the Golgi [1]. Furthermore, whereas N-linked glycosylation always consists of a common core structure of two GlcNAc and three Man residues, where a GlcNAc is always found covalently attached to the asparagine side chain, O-linked glycans attached to proteins can begin with the covalent attachment of a variety of monosaccharides via either an alpha or beta linkage, which can vary by the protein (Table I) [2]. O-linked glycans typically consist of a simple monosaccharide sugar added to a polypeptide, but some can be extended by Golgi glycosyltransferases into linear and branched glycans [50], including mucins and glycosaminoglycans. A

majority of O-linked glycans are found in extracellular, cell surface or intracellular membrane areas, but a significant O-linked modification is also found in the cytosolic and nuclear compartments.

TABLE I: TYPES OF O-LINKED GLYCOSYLATION AND WHERE THEY ARE FOUND

Sugar Residue	**Amino Acid**	**Alpha or Beta Linkage**	**Glycoprotein Examples**
GalNAc	Serine/Threonine	Alpha	Mucins [51]
GlcNAc	Serine/Threonine	Beta	Cytosolic and nuclear proteins [32]
Man	Serine/Threonine	Alpha	α-dystroglycan [52-53]
Glu	Serine	Beta	Notch [54]
Fuc	Serine/Threonine	Alpha	Thrombospondin [55]
Xyl	Serine	Beta	Proteoglycans; heparin [56-57]
Gal	Hydroxylysine	Beta	Collagen [58]
Glu	Tyrosine	Alpha	Glycogenin (autoglycosylation) [59]

Although O-linked glycans mostly consist of single sugar or oligosaccharide additions to target glycoproteins, these glycan chains also undergo extensive processing in the Golgi to form branched and lengthy linear glycans (Figure 2). Mucins, as well as proteins containing mucin-like domains, are plasma membrane or secreted proteins that directly contribute to the cellular glycocalyx and extracellular matrix, respectively [60-63]. Formation of mucin-type O-glycans begins with the addition of a GalNAc residue to select serine or threonine residues within a target protein, yielding the Tn antigen [64]. Once this is formed, a variety of Golgi-resident

glycosyltransferases modify the structure further, creating a vast array of branched O-linked glycan structures [65]. Another set of extracellular matrix proteins that are heavily glycosylated are the proteoglycans, which contain long linear O-linked glycosaminoglycan (GAG) chains. GAG chains are attached to serine residues and consist of a common core of four sugars ($XylGal_2GlcA$) synthesized in the cis-Golgi, then are extended by disaccharide units by glycosyltransferases and further modified by sulfotransferases [1, 66]. These O-linked glycan modifications, similar to N-liked glycosylation, are necessary for mammalian development and survival, as mouse knockout studies indicate that a lack of the ability to form any of these structures leads to death of the mouse during embryonic development due to a series of major structural development defects, including aberrant formation of the respiratory system (mucins), as well as epithelial and mesodermal cell dysfunction (GAGs) [33, 67-73].

Another O-linked sugar modification of noteworthy mention is the reversible modification of over one-thousand nuclear and cytosolic proteins singly with GlcNAc on serine or threonine amino acids. This process is known as O-GlcNAcylation and is mediated by two enzymes—uridine diphospho-N-acetylglucosamine: peptide-β-N-acetylglucosaminyltransferase (OGT), which adds GlcNAc to target proteins, and O-GlcNAcase, which removes the GlcNAc [74]. Similar to other mouse studies described above, O-GlcNAcylation has been shown to once again be necessary for mammalian development and survival, as mice that are null for OGT lose embryonic stem cell viability, leading to death [75]. The importance of O-GlcNAcylation can also be seen from numerous studies that have identified many functions of this modification, including the modulation of cellular signaling pathways in response to nutrients, modulation of protein trafficking and degradation, modulation of muscle contraction and cytoskeleton formation, protection of proteins during times of chronic stress or trauma, and a variety of roles in gene transcription and translation [reviewed in 32]. Interestingly, protein O-GlcNAcylation demonstrates a high level of crosstalk with protein phosphorylation, as both processes can occur on serine and threonine residues and in many cases, they occur on the exact same residues in one protein [76-78]. Reversible modification of a protein amino acid with GlcNAc and phosphate alters the activity of that particular protein depending on which of those molecules is bound. The Tau protein, which is a known phosphorylated protein that forms neurofibrillary tangles in Alzheimer's disease, has been found to be highly O-GlcNAcylated and this has an effect on the protein's

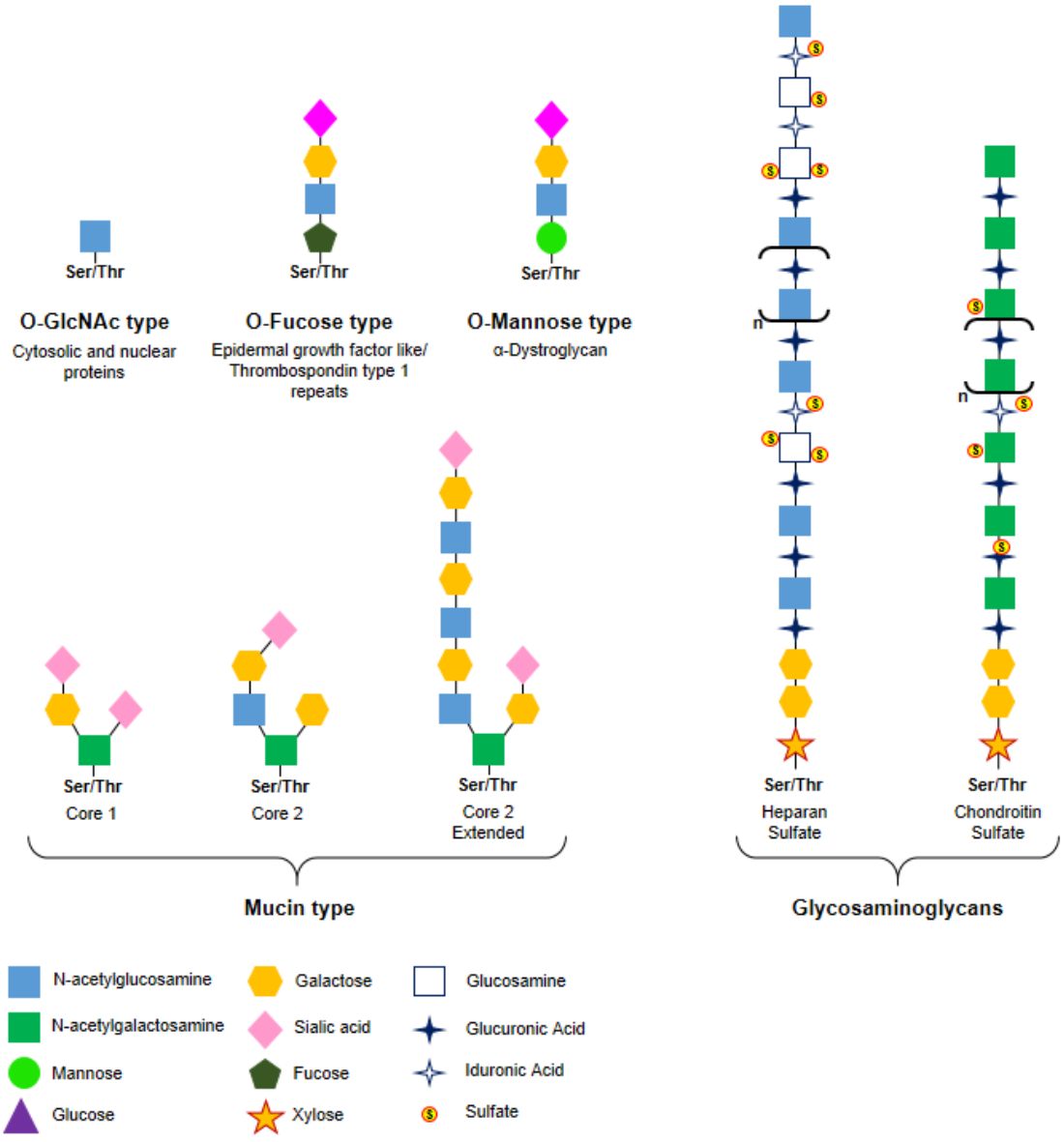

Figure 2. Examples of O-linked glycosylation. O-glycosylation is the glycosylation of target proteins on select serine or threonine residues. Unlike N-linked glycans which always begin with GlcNAc attached directly to an arginine residue, O-linked glycan chains can begin with of several different monosaccharides, including GlcNAc, Fuc, Man, GalNAc, or Xyl. O-glycosylation can be as simple as the addition of a single monosaccharide (O-GlcNAc), or can be extended into branched (mucins) or linear (O-fucose type, O-mannose type, glycosaminoglycans) glycan chains.

phosphorylation state [79]. A brain knockout of OGT leads to the hyper-phosphorylation of Tau utilizing sites of GlcNAcylation and neuronal death [80], whereas inhibition of GlcNAcase in rats leads to the reduction of Tau phosphorylation at sites determined to be critical for Alzheimer's disease pathology [81]. Another important function for GlcNAcylation that is coming to be realized is its role in the progression of diabetes. Although its role is still being fully deciphered, the modification of several insulin signaling pathway proteins with GlcNAc serves to alter the overall signaling cascade. Higher glucose levels have correlated with increases in O-GlcNAcylation levels [82-83]. As an example, it has been shown that treatment of NIH 3T3-L1 adipocytes with glucose leads to the O-GlcNAcylation of glycogen synthase, leading to a reduction in this enzyme's activity and the subsequent reduced uptake of glucose and its storage as glycogen [84]. Furthermore, insulin-stimulated phosphorylation of Akt at threonine-308 is inhibited in the presence of elevated GlcNAc levels, leading to a reduction in glycogen synthase kinase-3β phosphorylation and activity [85]. As a final note, GlcNAc has been demonstrated to modify a variety of oncogenes and tumor suppressors, including c-Myc [86], retinoblastoma [87], β-catenin [88], and estrogen receptors [89-90], and may play roles in the etiology of a variety of human cancers.

Disorders of N- and O-linked glycosylation

Congenital disorders of N-linked glycosylation

The inability to create complete N- or O-liked glycans on target glycoproteins, or the inability to successfully degrade glycans, constitute a large group of disorders that result from congenital defects in various enzymes along the various glycan biosynthetic or lysosomal degradation pathways [reviewed in 40,91]. In terms of N-linked glycans, congenital disorders of glycosylation (CDGs) are broken into two distinct groups—those resulting in the inability to fully synthesize the lipid-linked precursor oligosaccharide (CDG-I), and the inability to create complex N-glycan chains from the core oligosaccharide (CDG-II). In CDG-I, the net result is a decrease in the number of normal N-glycan structures, whereas the result of CDG-II defects is the creation of simpler, shorter chains without affecting the total number of N-glycans. Most of these CDGs are autosomal recessive, and are the result of the failure to synthesize adequate levels of a nucleotide sugar donor or the malfunction of a particular glycosyltransferase, but the overall clinical picture is not very different. Patients with these disorders tend to exhibit

multiple organ failure, including an array of neurological pathologies, immunological diseases, and liver pathology [91]. These pathologies can become serious early on in life, as demonstrated in the case of phosphomannomutase deficiency (CDG-Ia) that leads to inadequate levels of GDP-Man required for the synthesis of the $Glc_3Man_9GlcNAc_2$ lipid linked precursor oligosaccharide, 20% of infants die within the first year of life and those who live, have mental retardation along with peripheral neuropathy and kyphoscoliosis [92].

Hypomannosylation of α-dystroglycan

A group of degenerative disorders with a key O-glycosylation defect are the muscular dystrophies. These are congenital disorders that result in progressive degeneration of muscle fibers beginning at an early age, resulting in skeletal muscle wasting and atrophy [reviewed in 93-94]. At the center of a majority of these disorders is the dystrophin-glycoprotein complex (DGC), which consists of multiple glycosylated proteins and serves to mediate the interaction between the muscle fibers, the extracellular matrix, cytoskeleton, and cell membrane and thus provide mechanical stabilization for the muscle [reviewed in 95]. One of the proteins within the DGC, known as dystroglycan, is heavily O-glycosylated on its alpha subunit with O-linked Man residues which serve to bind with extracellular matrix proteins, including laminin, agrin and perlecan [96]. These interactions are absolutely essential for connecting the cellular cytoskeleton with the extracellular matrix [97]. Hypomannosylation of α-dystroglycan and failure to further modify the O-Man glycan is believed to destabilize the DGC and is the featured defect in a number of congenital muscular dystrophies including Walker-Warburg syndrome and muscle-eye-brain disease, both of which contain a variety of ocular abnormalities, structural brain malformations, and mental retardation [98-99]. Furthermore, the hypomannosylation of α-dystroglycan has been noted to disrupt the functioning of heart muscle and result in a series of cardiomyopathies [100].

Lysosomal storage diseases

When glycoproteins are no longer needed, they are sent to lysosome compartments for degradation of both their amino acids and glycan chains. When a genetic defect in a select lysosomal enzyme is present, the protein or glycan serving as a substrate for the defective lysosomal enzyme does not get fully degraded and builds up in the

lysosome. This results in the secondary inhibition of other catabolic enzymes and permeases that are not deficient, resulting in a lysosomal storage disease (LSD) with the inability to degrade multiple proteins, oligosaccharides, lipids and glycosaminoglycans [101-103]. LSDs occur in about one out of every 100,000 live births across the world without respect to race and ethnicity. To date, numerous LSDs that result in the inefficient breakdown of glycan chains have been documented [reviewed in 40]. These include the inability to degrade mannose (mannosidosis), fucose (fucosidosis), sialic acid (sialidosis), and galactose (galactosialidosis). Clinical manifestations of LSDs are quite heterogeneous, affected multiple organs and systems, but in most cases neurological disorders will be present. As an example, in sialidosis, where the lysosome is unable to degrade α2, 3- and α2, 6-linked terminal sialic acid residues, patients reported to hospitals with seizures, overactive reflexes (hyperreflexia), and the inability to voluntarily coordinate muscle movements (ataxia) [104-105]. Furthermore, in galactosialidosis, adult patients present with ataxia, mental retardation, and the deterioration of neurons [40].

Mannose-6-phosphate and its necessity for lysosomal protein degradation

A noteworthy example of LSDs are the autosomal recessive mucolipidoses (ML) II (I-cell disease)/III (pseudo Hurler polydystrophy), in which the failure of a key glycosylation event on newly synthesized lysosomal hydrolases results in patients exhibiting dwarfism, joint stiffness, skeletal abnormalities, enlarged heart and liver, and mental retardation [106]. In the case of ML II, the symptoms become extremely severe in the first decade of life, with death typically resulting between five and eight years of age due to congestive heart failure or persistent pulmonary infections [107]. Under normal physiological conditions, newly synthesized lysosomal hydrolases are folded and modified in the ER and Golgi, but then need to be specifically targeted to lysosomal compartments. In eukaryotes, this is accomplished through the modification of lysosomal enzymes with mannose 6-phosphate (M6P), which is utilized as a recognition marker for lysosomal targeting [reviewed in 108]. The modification of lysosomal hydrolases with M6P occurs in two steps—first, following the addition of high-mannose N-glycans on target proteins in the ER, GlcNAc phosphotransferase catalyzes the transfer of GlcNAc 1-phosphate to the C6 position of select α1, 2-linked mannose residues on the high-mannose oligosaccharides [109-112]; secondly, the GlcNAc residue is removed by N-acetylglucosamine 1-phosphodiester N-acetylglucosaminidase (NAGPA), which leaves

an exposed phosphate on the C6 position of a mannose residue [113, 114]. The resultant M6P is then recognized by mannose 6-phosphate receptors, and lysosomal proteins containing this recognition marker are shuttled to early or late endosomes where they can disassociate from M6P receptors and are then transported further to the lysosomes [reviewed in 115]. In ML II, genetic mutations in the *GNPTA* gene results in the absence of GlcNAc phosphotransferase, abolishing the formation of M6P on lysosomal enzyme N-glycan chains and resulting in the inability of these enzymes to reach the lysosome, and their secretion from cells [106]. Fibroblast cells from ML II patients characteristically demonstrate the presence of enlarged lysosomes that are filled with undigested proteins [116]. Patients with ML III exhibit the same symptoms as ML II patients but in a milder form, since this disease is genetically heterogeneous and GlcNAc phosphotransferase activity is only reduced and not absent [117].

The modification of lysosomal enzymes with M6P was the first described example of glycosylation that is considered to be protein-specific, and demonstrates that such glycosylation events are especially critical for maintaining normal mammalian physiology. To date, several other examples of protein-specific glycosylation have been documented and will be discussed in the following section.

Determinants and specificity of protein glycosylation

Although glycan chains consist of a common "core" set of sugars, such as the $GlcNAc_2Man_3$ structure that is common of all hybrid and complex N-glycans, a very large spectrum of N- and O-linked glycans are created. What are the criteria that lead to the creation of a specific glycan chain on a specific protein? "Core" oligosaccharides are typically expressed in many cell lineages. However, it is how these core sugars are terminally modified that leads to the great diversity of mammalian glycosylation products created, and these terminal glycan linkages are typically determined by the cell lineage and is typically tissue-specific [33]. Furthermore, a variety of factors within the secretory pathway play a critical role in what glycans will be produced. These include the spectrum of glycosyltransferases present in a particular cell at a certain time and in a certain amount, the synthesis of nucleotide sugars, the presence of Golgi membrane transporters for those nucleotide sugars to enter the Golgi lumen, as well as the structure and organization of the Golgi membranes [1]. Furthermore, the pH of the Golgi

lumen has been shown to alter the cellular glycosylation levels, as the higher the pH, the slower glycoproteins are trafficked through the secretory pathway, resulting in truncated glycosylation of these proteins [118, 119].

Some proteins, and specific domains within them, are selectively glycosylated with specific classes of glycan structures, which have been shown to play critical roles in development and physiology. The first example of protein-specific glycosylation is the modification of lysosomal enzymes with mannose 6-phosphate, which was discussed in detail above. Another example of protein-specific glycosylation is the addition of fucose residues to the EGF domain of Notch family proteins. EGF domains are cysteine-rich sequences of 30-40 amino acids that are found on many cell surface and secreted glycoprotein and are involved in protein-protein interactions [120]. Serine and threonine residues within the EGF that are between the second and third cysteine residues are modified first with Fuc [121, 122], followed by chain extension to form a tetrasaccharide (Sia-Gal-GlcNAc-Fuc-Serine) that has been shown to modulate Notch-ligand interactions during development that have a role in cell fate decisions [123]. In mouse studies, EGF glycosylation has also been shown to be necessary for maintaining normal hematopoiesis [124]. A second example is the glycosylation of thrombospondin type 1 repeats (TSRs). TSR domains are found in numerous cell surface proteins and consist of tandem repeats of 50-60 amino acids with disulfide bonds formed by six conserved cysteine residues [2]. TSR domains can be glycosylated on serine or threonine residues with a Fuc sugar using a different glycosyltransferase than in EGF domains, or modified with Man on a tryptophan residue via a carbon-carbon bond [125, 126]. Recent mouse knockout studies indicated that glycosylation of TSR domains is critical for several developmental processes, including the epithelial to mesenchymal transition and cell patterning [127]. The third well-studied example of protein-specific glycosylation is the modification of α-dystroglycan with mannose residues, which has been discussed above.

Another example of protein-specific glycosylation is the modification of neural cell adhesion molecule with polysialic acid. This modification occurs on a very select group of glycoproteins and has been shown to have both necessary and detrimental effects over the course of mammalian life. Despite a thorough understanding of the biological effects of polysialic acid, the mechanism by which this modification occurs has not been fully deciphered. The roles of polysialic acid in mammalian development and life will be discussed in detail below, and the work

presented in subsequent chapters aims to elucidate the biochemical mechanism of polysialic acid addition onto target glycoproteins.

Polysialic acid

Polysialic acid (polySia) is a linear homopolymer of α-2, 8-linked N-acetylneuraminic acid (sialic acid) residues that is synthesized on the termini of N- or O-linked glycans found on select glycoproteins (Figure 3). The synthesis of polySia occurs in the Golgi apparatus and is catalyzed by two human polysialyltransferase (polyST) enzymes—ST8Sia II (STX) and ST8Sia IV (PST). The polySTs are Golgi localized glycosyltransferases found in the *trans* cisternae and the *trans* Golgi network [128]. In order to synthesize these linear chains of sialic acid, the polySTs require (1) an acceptor α2, 3- or α2, 6-linked sialic acid residue terminating a glycan chain, and (2) an activated sialic acid residue found on the donor CMP-sialic acid. The resulting polySia chains have been shown to extend to approximately 57 sialic acid residues in the mouse brain, and over 100 units in human tumor cells [129, 130]. The process of substrate polysialylation is considered highly protein-specific, as to date there are only seven known glycoproteins that are targeted for modification with polySia. These substrates are the neural cell adhesion molecule (the polySTs' major substrate) [131], neuropilin-2 [132], synaptic cell adhesion molecule 1 [133], the alpha chain of the voltage-sensitive sodium channel [134], a CD36 receptor found in human milk [135], and the polyST enzymes themselves, in a process known as autopolysialylation [136, 137]. The modification of glycoproteins with polySia has been shown extensively to play necessary roles in mammalian embryonic development as well as both critical and detrimental roles in the adult. Most of the studies that deciphered these important functions of polysialylation came on work utilizing the polySTs' major substrate, neural cell adhesion molecule, and these studies will be detailed in the following sections.

Neural cell adhesion molecule – the major polysialylated substrate

The neural cell adhesion molecule, or NCAM, is a cell surface glycoprotein whose major and most well-studied function is to mediate cell-cell adhesion through interactions with NCAM molecules on an opposing cell surface. There are three isoforms of NCAM that are named based on their approximate molecular weight (NCAM-

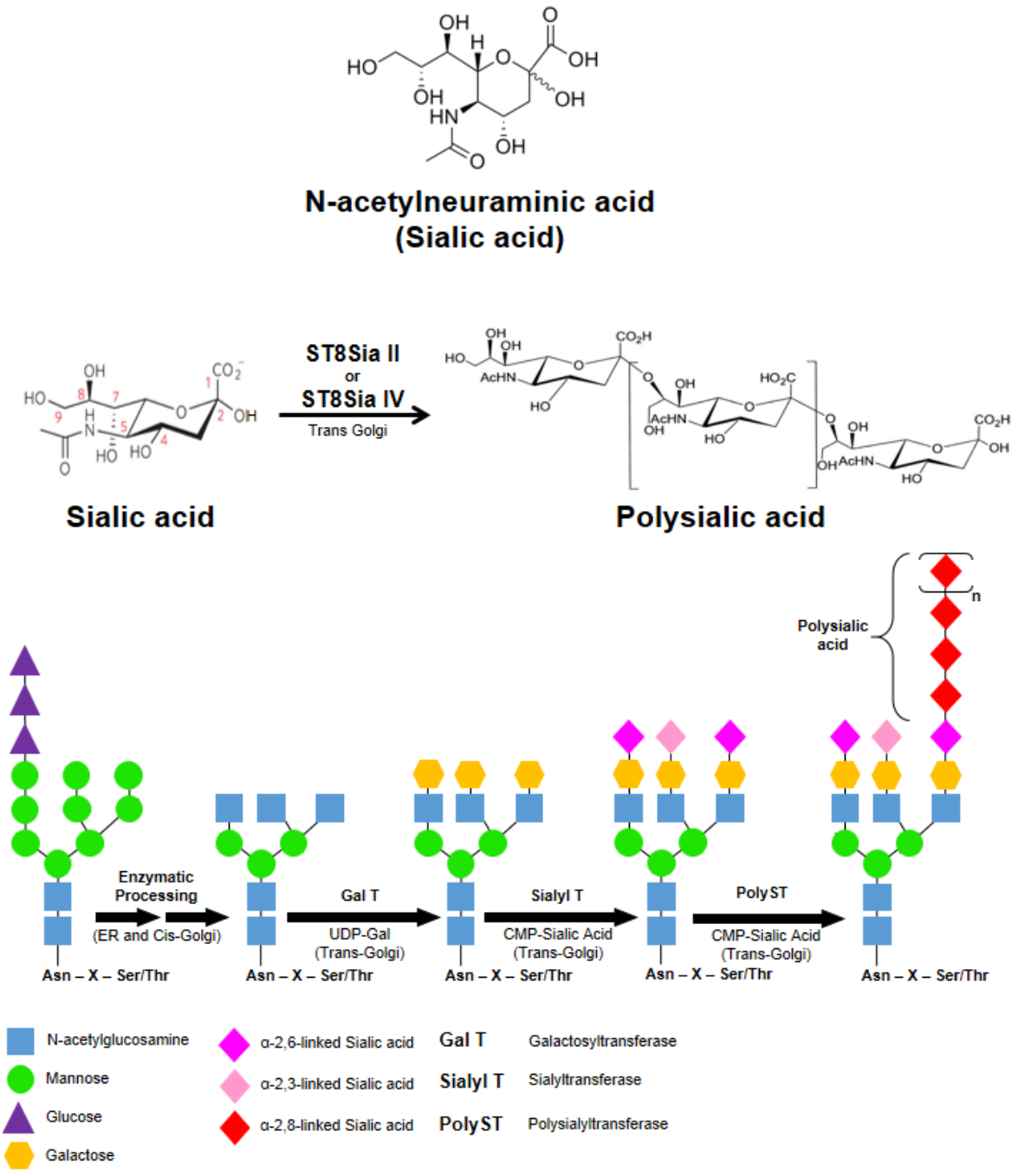

Figure 3. Overview of polysialic acid synthesis. Polysialic acid is a linear homopolymer of α2, 8-linked sialic acid residues that is synthesized in the Golgi apparatus by ST8Sia II and ST8Sia IV on an α2, 3- or α2, 6-linked sialic acid residue terminating a glycan chain.

120, NCAM-140, and NCAM-180) and are created through alternative splicing of pre-mRNA that is encoded by one *NCAM* gene (Figure 4) [138]. In their extracellular portions, all three isoforms of NCAM contain five immunoglobulin-like domains (Ig1 – Ig5) followed by two fibronectin type III repeats (FN1 and FN2). The isoforms then differ in the way in which they are attached to the plasma membrane, containing either a transmembrane region and cytosolic tail (NCAM-140 and NCAM-180) or a GPI-anchor (NCAM-120). NCAM has been previously shown to form homophilic interactions with NCAM molecules on other surfaces as well as on the same plasma membrane, and it has been postulated that when NCAM is not forming *trans*-homophilic interactions with NCAM on an opposing surface, it is forming *cis* interactions with other NCAM molecules on the same plasma membrane [139]. The exact mechanism by which NCAM forms *cis*- and *trans*- homophilic binding interactions has always been a subject of debate, and many studies have led to the formation of multiple theories concerning which domains mediate these interactions [140-147]. However, crystallographic analyses seem to indicate that *cis*-homophilic NCAM binding is mediated by the first two Ig domains (Ig1 and Ig2), whereas *trans*-homophilic NCAM binding may involve interactions between residues within the Ig2 and Ig3 domains, and could include residues in Ig1 as well [148].

Polysialic acid modulates neural cell adhesion molecule cell-cell adhesion properties

NCAM contains six asparagine residues within the Asn-X-Ser/Thr consensus sequences required for N-linked glycosylation—site 1 is found in the Ig3 domain, sites 2 and 3 are in Ig4, and sites 4, 5 and 6 are located in Ig5 [149]. PST and STX modify NCAM with polySia on glycans attached to asparagine sites 5 and 6 in the NCAM Ig5 domain [150]. Since each sialic acid residue has a net negative charge, the addition of long chains of polySia to NCAM introduces a large amount of negative charge to the region surrounding the NCAM molecule, allowing for the coordination of water molecules and increasing the hydrodynamic radius of NCAM (Figure 5) [151]. This prevents two NCAM molecules from coming close enough to each other to functionally interact, leading to a decrease in cell-cell adhesion [152, 153], an increase in intermembrane repulsion [154], and the promotion of lateral diffusion of NCAM within the plasma membrane [155]. Therefore, polySia serves as an anti-adhesive glycan that prohibits the normal cell-cell adhesive functions of NCAM and other adjacent adhesion molecules [152, 155].

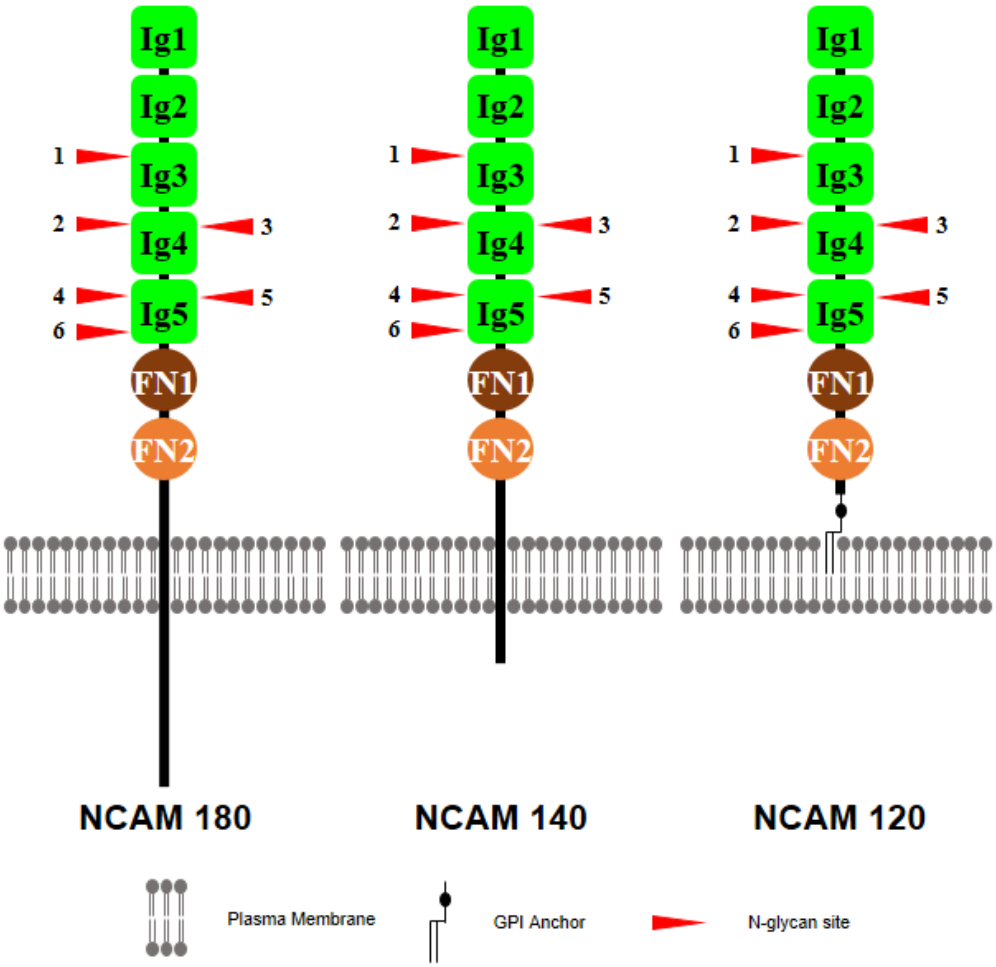

Figure 4. Isoforms of neural cell adhesion molecule. The three isoforms of NCAM are named after their approximate molecular weight. NCAM 120, NCAM 140, and NCAM 180 have identical extracellular domains, including five immunoglobulin-like domains (Ig1-5), two fibronectin type 3 repeats (FN1 and 2), and six N-glycosylation sites. They differ on the length of their cytosolic tails (NCAM 140 and NCAM 180) or presence of a GPI anchor (NCAM 120).

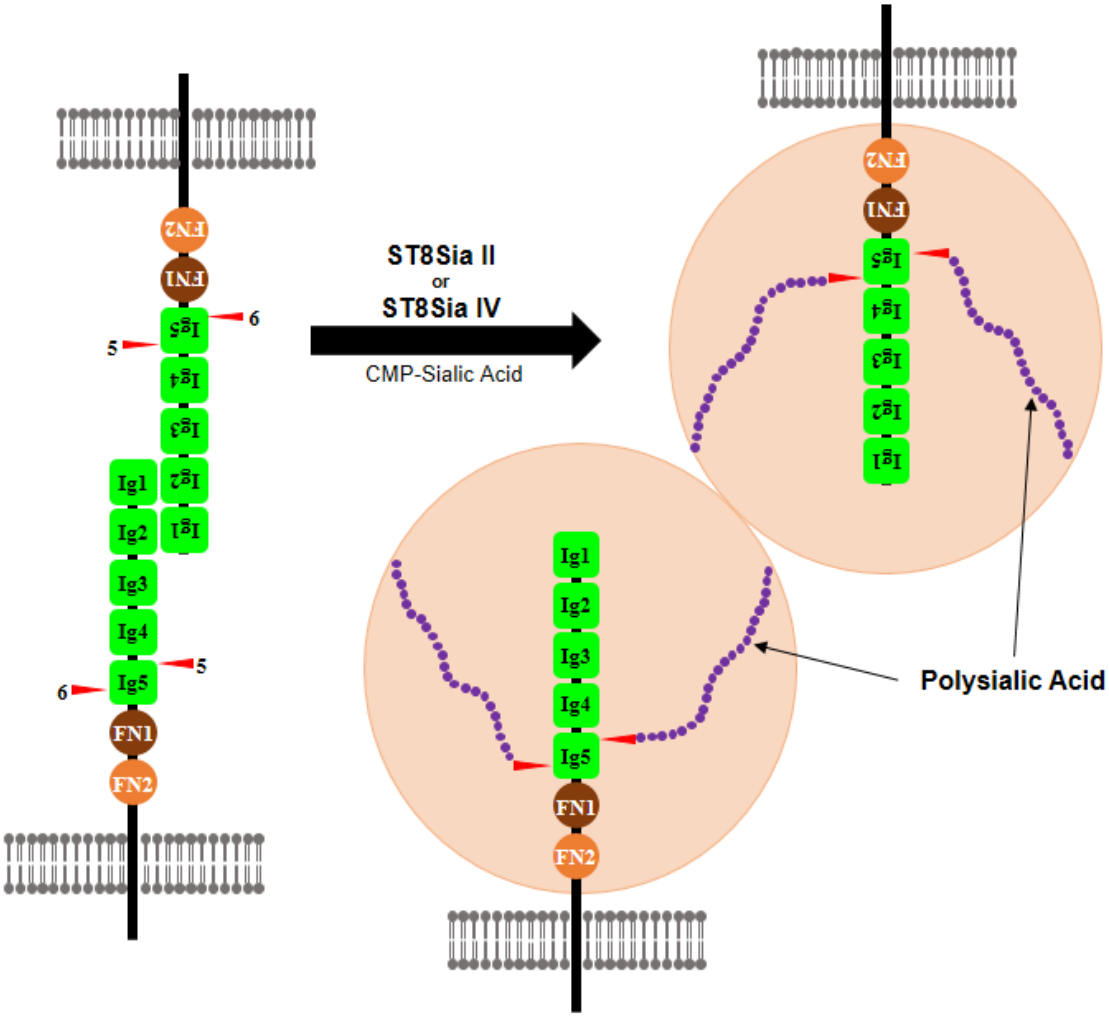

Figure 5. Effect of polysialic acid on NCAM cell-cell adhesion properties. Synthesis of polysialic acid on N-linked glycan sites five and six in the Ig5 domain introduced a large amount of negative charge surrounding the NCAM molecule. This increases the hydrodynamic radius of NCAM (*pink ovals*) and prevents the homophilic interaction of NCAM molecules on opposing cell surfaces.

Polysialic acid modulates cell signaling

Even though NCAM does not possess any known direct catalytic activity that would be capable of initiating downstream signaling pathways, numerous studies have demonstrated that NCAM is capable of mediating neurite outgrowth by affecting a variety of signal transduction cascades through a series of intracellular and extracellular binding partners [reviewed in 156]. A well-studied example of NCAM-mediated signal transduction via downstream proteins is the involvement of a Src family nonreceptor tyrosine kinase, Fyn [157]. Beggs *et al.* [157] found that cortical neurons from $Fyn^{-/-}$ mice displayed stunted neurite extension when grown on NCAM-expressing fibroblasts. This finding was unique, as knockouts of other Src family members did not affect NCAM-mediated neurite outgrowth. Furthermore, constitutive interaction between NCAM and Fyn was discovered through co-immunoprecipitation analysis [158, 159]. In more recent studies, Fyn phosphorylation and resultant activity were found to be lower in NCAM-deficient mice than in wild-type mice [160], and that clustering of NCAM on the neuron cell surface induces high levels of Fyn phosphorylation [158]. Taken together, these results demonstrate that NCAM is capable of mediating cellular signaling by affecting the activity of downstream effectors. More recently, it has become apparent that modification of NCAM with polySia further impacts these signal transduction events, and that many of the effects might involve alterations in signaling as well as adhesion. Some of the main studies demonstrating the effects of polySia on NCAM signaling properties are summarized here.

Fibroblast growth factor receptor

The fibroblast growth factor receptor (FGFR) is found on the plasma membrane and consists of three extracellular immunoglobulin-like domains, a transmembrane region, and an intracellular domain that has tyrosine kinase activity. FGFR is activated by forming a dimer with another FGFR molecule, leading to transphosphorylation of its tyrosine kinase domains and transduction of an extracellular signal to the nucleus to promote neurite outgrowth [156]. Receptor dimerization occurs upon binding to its main ligand, the fibroblast growth factor (FGF).

Utilizing surface plasmon resonance and NMR analyses, it has been demonstrated that an FG loop in the FN2 domain of NCAM directly binds to the extracellular domain of FGFR [161]. This interaction has been shown

by multiple studies to promote NCAM-mediated signaling by promoting FGFR transphosphorylation and subsequent activation [161, 162]. Furthermore, the NCAM-FGFR interaction is absolutely critical for NCAM signaling ability, as pharmaceutical inhibition of FGFR in cerebellar granular neurons, inhibition of FGFR in cells with FGFR antibodies, or expression of dominant negative FGFR receptors in PC12 cells leads to a significant reduction in the ability of NCAM to promote neurite outgrowth [162-165]. From studies examining the patterns of activated adapter proteins, it was determined that NCAM activates FGFR in a mechanism that is different from FGF [166]. Additionally, the affinity of the NCAM-FGFR interaction is much weaker than that of FGF-FGFR, but the activation of FGFR by NCAM is biologically significant because there is a much higher concentration of NCAM molecules at the cell surface at any one time than there is FGF [139].

The presence of polySia on NCAM is postulated to play a significant role for NCAM-mediated FGFR activation. When NCAM is not polysialylated, it is believed that NCAM on a cell surface will form tight interactions with NCAM molecules on an opposing cell surface, resulting in the formation of a "zipper" that does not allow enough room for interaction with other molecules [156]. In this scenario, FGFR is unable to be activated by NCAM. When NCAM is polysialylated, the resultant increase in NCAM's hydrodynamic radius negatively regulates its ability to form these *trans*-homophilic interactions, leaving NCAM molecules less densely packed and in a position to activate FGFR signaling [156].

Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family found within the central nervous system. It is a secreted protein that has been shown to be critical for the survival, growth and differentiation of neurons and synapses, which is accomplishes through binding to and activating its receptor [167-170]. It has also been shown that BDNF is important for long-term memory [171]. Work from Vutskits *et al.* [172] indicated a critical role for polysialylated NCAM for the proper function of BDNF. When polySia was removed from NCAM in cultured cortical neurons using EndoN, or inactivated using an antibody against polySia, cortical neuron survival was significantly reduced, as was intracellular signaling through BDNF-stimulated signaling pathways. The loss of BDNF signaling could be replenished by the administration of excess, exogenous BDNF. These data suggested that

polySia on NCAM plays a role in BDNF-mediated intracellular signaling by potentially sensitizing neurons to BDNF.

Platelet-derived growth factor

In the mammalian nervous system, directed migration of oligodendrocyte precursor cells (OPCs) is critical for myelin formation in the central nervous system during neuronal development as well as during neuronal injury in the adult [173-175]. The migration of OPCs has been previous shown to migrate toward its targets following a chemical gradient of platelet-derived growth factor (PDGF) [176]. Work from Zhang *et al.* [177] suggests a role for polySia attached to NCAM in the response of OPCs to PDGF. Utilizing migration studies using gradients of PDGF, they found that removal of polySia from NCAM altered the migratory pathways of OPCs. With polySia-NCAM present on OPCs, these cells exhibit a biased migration toward the PDGF source, whereas random migration is demonstrated when polySia is inactivated or removed from NCAM. Although the direct role of polySia-NCAM on PDGF-mediated signaling and migration is still being determined, it was suggested from these results that polySia sequesters PDGF near the cell membrane, stimulating signaling and directional migration by increasing OPC's sensitivity to this migratory cue [178].

Mitogen-activated protein kinase pathway

Numerous studies have suggested that in response to NCAM stimulation, the terminal mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 are phosphorylated and activated via MEK and Ras, which in turn are activated through pathways that initiate with FGFR [156, 179]. The net outcome is NCAM-mediated neurite extension. Using neuroblastoma tumor cells, Hildebrandt *et al.* [180] suggest a negative role of polySia in modulating neurite outgrowth in response to NCAM. Utilizing SH-SY5Y neuroblastoma cells, they found that NCAM promotes neuritogenesis in response to retinoic acid stimulation through activation of ERK. However, in the presence of polySia-NCAM, signaling via ERK is altered and neurite outgrowth does not occur.

NCAM is a cell surface glycoprotein that is known to interact with FGFR and Fyn to activate these latter proteins, leading to cellular signaling cascades that promote neurite outgrowth. Furthermore, the presence of polySia

can alter NCAM signaling properties. A common feature of NCAM signaling is an activation of the MAPK pathway. However, the precise mechanism by which a cellular signal is transmitted from NCAM-activated FGFR or Fyn to Ras/Raf/MEK/ERK is still emerging. Numerous studies point to a variety of intracellular ion proteins and pathways that may have a role in NCAM signaling through to ERK, including a rise in intracellular Ca^{2+} in response to NCAM stimulation [181-184], increases in protein kinase A and C activity [182, 185], and activation of the phosphatidylinositol 3-kinase/Akt and cyclic guanosine monophosphate pathways [186, 187].

PolySia and sequestration of extracellular signaling factors

Although the precise mechanism by which polySia modulates cell signaling is not fully understood, several recent studies have indicated that polySia can directly bind to FGF and BDNF [188-190]. From these works, it has been postulated by Kanato *et al.* [190] that polySia serves as a "sink" for neurotrophic factors, binding to them and thus concentrating them near the cell surface, thus promoting the activation of a cell surface receptor by its ligand and hence altering overall signaling, regardless of whether the resultant signaling is desirable or not.

Expression patterns of polysialic acid

PolySia is first expressed on embryonic day 8-8.5 in the mouse and remains expressed for the entirety of pre-natal development [191]. Approximately three weeks after birth, the expression of polySia disappears from a majority of neural regions, which coincides with the completion of major morphogenic events within the mouse, including cell proliferation, migration, neurite extension and synapse formation [192-195]. In the adult animal, consistent polySia expression is restricted to select regions of the brain where the formation of new neuronal tracts is necessary [reviewed in 196]. The regulation of polySia expression during development is explained by the expression patterns of the two polySTs. PST is moderately expressed during embryonic brain development and remains expressed in specific portions of the adult brain, whereas STX is more highly expressed in the embryo and neonate and then declines substantially in the adult [197]. The appearance of polySia during mouse embryonic development and its subsequent disappearance postnatally raises the question as to the role of polySia in development.

Polysialylated NCAM is necessary for the development of the mammalian nervous system

To determine the role of polySia in development, Weinhold *et al.* [198] simultaneously deleted ST8Sia II/STX and ST8Sia IV/PST to create a polySia-negative mouse. These double knockout mice exhibited abnormalities in major brain fiber tracts, hydrocephalus, impaired postnatal growth, and death of these mouse occurred 3-4 weeks after birth. However, when NCAM was subsequently deleted ($STX^{-/-}PST^{-/-}NCAM^{-/-}$ triple knockout mice), all of the mentioned effects were reversed, and the mice were viable. These results suggest that without polySia, there is an abnormal gain of NCAM function causing abnormal neuronal development. Results from Hu *et al.* [199] demonstrate that the phenotype observed in these mice may be a result of a failure of migration of neurons during development. Mice treated with endoneuraminidase N (Endo N), an enzyme that specifically cleaves α-2, 8-linked polySia, showed that interneuron progenitor cells in the subventricular zone did not reach to the olfactory bulb, where they normally differentiate. Instead, the loss of polySia caused them to inappropriately adhere before reaching the olfactory bulb, resulting in their differentiation into interneurons prematurely and in the wrong place. In total, these results indicate that polySia synthesized by the polySTs during nervous system development is absolutely necessary because it serves to "mask" NCAM so that NCAM-NCAM contacts are made only in a highly organized, time- and site-specific manner to ensure proper migration of neurons and promote correct nervous system development.

Expression of polySia in the normal adult brain

After being expressed at high levels throughout embryonic development, the levels of polySia diminish in the adult, but remain constitutively expressed in areas of ongoing neuronal plasticity. Some of the key locations of ongoing neuronal plasticity and the roles of polySia in each location are detailed here below.

<u>Hippocampus</u>

Numerous studies have demonstrated the presence of polySia in regions within the adult brain that are associated with memory formation, including the hippocampus, dentate gyrus, amygdala, piriform cortex and the neocortex [193, 200-202]. Removal of polySia from NCAM on hippocampal progenitor neurons using Endo N *in*

vivo did not prevent the differentiation of these cells, but did promote the abnormal clustering of these progenitors in the dentate gyrus portion of the hippocampus, preventing their migration from this region [203]. Furthermore, adult rats injected with Endo N exhibited a significantly impaired ability to successfully perform in the Morris water maze, suggesting the inability of these rats to acquire and retain spatial memory [204]. Furthermore, treatment of hippocampal slices with Endo N in order to study the effects of polySia on long-term potentiation, which is an underlying phenomena promoting synaptic plasticity in learning and memory [205, 206], indicated that this process was greatly impaired in these mice [204]. Taken together, the constitutive presence of polySia in the adult hippocampus and associated regions promotes our ability to continuously form new memories, likely through the proper formation of new neuronal tracts and neuronal synapses [207].

Olfactory bulb

The ability of a mammal to smell a variety of odors is critical for identification of surroundings as well as warning us of surrounding dangers. To maintain our ability to smell, olfactory interneurons continuously migrate in chains through the rostral migratory stream from the subventricular nucleus, located in the wall of the lateral ventricle, to the olfactory bulb [208]. Removing polySia both *in vivo* and *in vitro* prevented the ability of olfactory interneurons from forming organized migrating chains, leaving these cells dispersed and disorganized near the subventricular nucleus [209]. Therefore, polySia promotes proper formation and migration of olfactory interneuron chains and hence maintains our ability to smell.

Hypothalamus

PolySia persists within the hypothalamus and associated neurohypophysis, which is associated with neuroendocrine functions [210]. Within these regions, polySia has been shown to be important for normal functioning of the ovarian cycle. In the pro-oestrous phase of the cycle, GnRH neurons have to extend projections from the pre-optic area in order to establish the appropriate contacts to promote hormonal release, whereas in the di-oestrous phase, these neuronal projections normally retract [reviewed in 196]. The expression of polySia correlates with this cycle, as levels of polySia are high during the pro-oestrous phase, when neuronal projections

are necessary, and levels are low during the di-oestrous phase [211]. Additionally, removal of polySia enzymatically completely blocks the ability of these neurons to send out projections, disrupting the entire ovarian cycle [212]. Furthermore, studies indicate a role for polySia in the proper migration of neurons of the supraoptic and paraventricular hypothalamic nuclei in order to allow for the proper secretion of oxytocin and antidiuretic hormone in response to lactation and dehydration, respectively [213-215]. Therefore, fluctuating levels of polySia in the hypothalamus promote the secretion of hormones at the times that they are needed.

Suprachiasmatic nucleus

The suprachiasmatic nucleus (SCN) houses the mammalian circadian clock, which is entrained through light input from the retina. The amount of polysialylated NCAM in the SCN greatly increases in response to light input from the retina [216], and cycles continuously through a twenty-four hour period, raising the question as to the role of polySia in establishing our circadian rhythms. To answer this question, several studies have enzymatically removed polySia from the SCN. When polySia is removed, generation and establishment of a circadian rhythm is impaired due to the inability to activate the Fos transcription factor within the SCN, as well as the inability to reset the circadian rhythm in the presence of light [216, 217]. Unlike in previous cases, there are no changes in innervation of the SCN by retinal neurons, and the precise mechanism by which polySia affects circadian rhythms is not fully understood [218].

Transient expression of polySia in the normal adult

PolySia is downregulated in a majority of tissues within the adult mammal except for a select few regions as detailed above. However, in some cases, the need arises for polySia in area that no longer require this glycan modification. The transient expression of polySia in the adult refers to the temporary re-expression of the polySTs and polySia in regions that usually do not express them after birth.

Brain and peripheral neuron regeneration

In the central nervous system (CNS), consisting of the brain and spinal column, it has been shown that cerebellar Purkinje cells have the ability to sprout new axons in a period of about three months following axotomy

[219]. Dusart *et al.* [220] demonstrated the need for polysialylated NCAM for the regeneration of these cells following an axotomy within the rat cerebellum. They found that in the intact cerebellum, polySia-NCAM is not found. However, polySia expression in reactive astrocytes surrounding the injury site was found to start increasing fifteen days after Purkinje cell axon injury, and expression peaked three months later. The location and timing of polySia expression correlated with the general period of gliosis that occurs following the injury of these cells, suggesting a role of polySia in axon sprouting, potentially to aid guidance of the growing axon.

Mouse studies demonstrate that polySia is homogenously expressed in the cell bodies of spinal ganglia neurons, which sprout to form neurons of the peripheral nervous system (PNS), beginning on embryonic day 12 and are necessary for the promotion of neurite migration and growth during development [221-223]. However, polySia is not found on the myelinating Schwann cells of the PNS, despite the presence of NCAM [224, 225]. Interestingly though, polySia on NCAM is upregulated in Schwann cells following nerve injury [226-228]. Work from Jungnickel *et al.* [229], in which the sciatic nerves of mice were crushed and analyzed before and post-injury, indicated a role of polySia in PNS nerve regeneration. Utilizing mice that had only one functional polyST allele, they found that following sciatic nerve crush, these mice exhibited fewer myelinated fibers and a slower regeneration rate than found in wild-type mice. Furthermore, the regeneration of sciatic nerve fibers was found to be stunted to a much greater extent in mice missing the ST8Sia II/STX allele, suggesting that both polySTs are required for proper nerve regeneration but that STX has a more prominent role. Taken together, the polySTs work in tandem to synthesize polySia in the PNS during times of nerve injury.

Spinal cord injury

Lesions of the spinal column interrupt neuronal connections between the CNS and the PNS, which become difficult to repair due to the inability of PNS dorsal root ganglia to cross over the dorsal root entry zone (DREZ) to reconnect with CNS targets [230-232]. The inability to form these connections after spinal column injury may be related to several factors, including the presence of reactive astrocytes at the site of injury or the lack of growth-promoting factors on the surface of astrocytes [233-236]. These hardships can lead to permanent loss of motor functions after the injury, which begs the questions as to potential treatments to aid in neuron regeneration. Several

studies have pointed to polySia as a potential therapeutic after spinal cord injury [237-240]. Ghosh *et al.* [237] demonstrated that implanting Schwann cells overexpressing polySia near a spinal cord lesion improved a mouse's recovery time as well as their overall motor function recovery as compared to mice receiving regular Schwann cells. This result was due to an increase in the migration capability of these modified Schwann cells, as those cells not overexpressing polySia remained confined to the site of injection. In two studies, Zhang *et al.* [238, 239] found that lentiviral delivery of PST to the spinal column increased astrocyte and Schwann cell infiltration of the lesion site and promoted more axons to cross the DREZ, thus improving recovery. Mehanna *et al.* [240] showed that treatment of mice with an induced spinal column transection with a polySia mimetic peptide through subdural infusion led to an increase in the total number of cholinergic and glutaminergic neuron terminals within the spinal cord, as well as more effective axon myelination. These studies indicate the need of polySia in times of neuronal injury and suggest that polySia could be a potential pharmaceutical treatment for these lesions.

Hyperthermia

Duveau *et al.* [241] provided evidence that an upregulation of polysialylated NCAM within the brain contributes to a phenomena known as "brain tolerance," which is the preconditioning of the brain with a subtoxic challenge in order to confer resistance to a potentially lethal challenge [242]. Exposure of mice to a heat stress induced both the production of heat shock protein 70 (HSP70) and polySia on NCAM. Exposure of mice to quercetin, and inhibitor of HSP70, also reduced the production of polysialylated NCAM following hyperthermia, whereas linoleic acid treatment, which upregulates HSP70, also increased levels of polySia-NCAM. Although the precise mechanism by which the rise and fall of polySia occurs is not fully known, it is suggested that polySia and HSP70 may work in tandem to provide an organism neuroprotection in the presence of hyperthermia.

Chronic stress

Persistence of an external stress activates a series of physiological cascades that can lead to alterations in brain structure and function, synaptic plasticity, as well as changes in hormonal release, cognitive and behavioral traits—all to help an organism adapt to its new environment [reviewed in 138]. Pham *et al.* [243] demonstrated a

change in polySia-NCAM levels in response to restraint stress within the mouse hippocampus. In response to chronic restraint stress for three weeks, mice displayed a noticeable shrinkage of their hippocampus, with a loss of cell proliferation and granule cell neurogenesis. Coinciding with these changes, there was a 40% increase in the amount of polysialylated NCAM in the hippocampus. These changes grew after six weeks of chronic stress, as exposed mice exhibited a 51% increase in polySia-NCAM as compared to unexposed mice. In these mice, the structure of the hippocampus is evidently changing, with overall shrinkage and attempts at neurogenesis occurring. With these observations, the authors suggest that polySia is temporarily upregulated in times of chronic stress to help aid in the restructuring of the hippocampus, as with structural rearrangement comes the need for cell migration and synapse formation. However, this issue is not fully deciphered yet and other theories for the upregulation of polySia in times of chronic stress exist. For example, it is theorized that polySia is serving as a neuroprotective agent, disconnecting NCAM-NCAM interactions at excitatory glutaminergic synapses that are overactive as a result of external stress [196].

Polysialylation in human disease

Thus far, the necessity of polySia in mammalian nervous system development, as well as the many critical and positive functions of polySia in the adult mammal have been detailed. From these details, it is shown that polySia is a key glycoprotein modification throughout life. However, it must also be noted that this is only half of polySia's story, as polySia levels have also been found in inappropriate levels, both increased and decreased, in several human disorders and cancer. In some disease states in which polySia levels are found to fluctuate, such as in Alzheimer's disease below, it will still be necessary to determine whether these polySia levels function in a neuroprotective sense or whether they are detrimental to the organism.

Schizophrenia

The brain of a patient with schizophrenia contains a plethora of neuronal architecture abnormalities within the frontal cortex, hippocampus, amygdala, fornix, anterior thalamic nuclei, and limbic cortex, including inappropriate orientation of hippocampal neurons and misplacement of NADPH-diaphorase-positive neurons in the

frontal and temporal cortices [244-248]. These histological features are considered to contribute directly to the formation of the schizophrenia pathology [249]. Patients with schizophrenia typically will present to the clinic with the inability to form normal emotions or process complex thoughts, and will have hallucinations and disorganized speech [250]. This is a genetic disorder that begins in one's teenage years and has a prevalence of 0.3-0.7% globally [251]. As the hippocampus is a location of ongoing neuronal plasticity and contains constitutive levels of polySia-NCAM through adulthood, Barbeau *et al.* [252] investigated polySia levels in the brains of schizophrenia patients. Interestingly, postmortem hippocampal samples from patients with schizophrenia exhibited up to a 95% reduction in the amount of polySia-NCAM as compared to the hippocampus samples from normal brains, suggesting that the altered architecture of hippocampi in schizophrenia may be a result of inappropriate lowered levels of polySia. In a further study, Isomura *et al.* [189] documented an E141K mutation within the STX enzyme in a patient with schizophrenia, which results in a significant reduction in the synthesis of polySia. Furthermore, they found that polySia has the ability to interact with dopamine. From these findings, it was postulated that in the brains of schizophrenia patients, reduced polySia levels prevents the effective sequestration of dopamine, thus impairing normal dopamine signaling. In schizophrenia, there are also other pathologies that have been documented, including imbalances of other cellular proteins within the hippocampus [253, 254] and inappropriate blood flow to the frontal cortex [255, 256], to name a few. Therefore, the precise role of polySia must still be elucidated, but it cannot be ignored given the necessity of this glycan chain in everyday hippocampal function.

Alzheimer's disease

Alzheimer's disease is the most common form of age-related dementia, to which there is no cure. Patients with this disease show varying short-term memory loss, irritation, confusion, aggression, and withdrawal from family, with death eventually occurring after the loss of various bodily functions. Within the brain, these patients exhibit an overall reduction in the number of neurons and synapses that are present [257-259], and the deposition of β-amyloid plaques and neurofibrillary tangles within the brain's grey matter, including within the hippocampus [260]. The role of polySia in Alzheimer's disease is still a matter of debate as to whether it is functioning in a positive or negative role. Mikkonen *et al.* [261] demonstrated that levels of polySia-NCAM within the dentate gyrus

of the hippocampus of Alzheimer's patients are significantly increased above the amount that is typically found in the normal brain. It has been suggested that since there are fewer neurons in the Alzheimer's brain, the excess polySia may contribute to the formation of new neuronal connections. On the contrary, it has been shown that as compensation for the presence of fewer neurons, new neurons aberrantly sprout within the hippocampus [262-264]. In this case, it is postulated that excessive polySia may promote abnormal connections, possibly contributing to the neurofibrillary tangles that are characteristic of the disease.

Epilepsy

Epilepsy is the result of the occurrence and chronic re-occurrence of seizures, characterized by episodes of abnormal, excessive hypersynchronous neuronal activity [265, 266]. Similar to Alzheimer's disease, epilepsy involves the loss of neurons and axonal sprouting within the hippocampus. The role of polySia within the hippocampus in the epileptic brain has been investigated and suggests that it may have roles in neuronal organization following seizures. Studies examining postmortem hippocampi samples from patients with temporal lobe epilepsy, in which seizures originate within the temporal lobes of the cerebrum, demonstrate a great increase in the amount of polySia-NCAM as compared to hippocampal slices from normal brain [267]. Furthermore, the increase in polySia-NCAM observed directly correlated with the duration of epilepsy, severity of hippocampal neuronal loss, and the density of mossy fiber sprouting, suggesting a role of polySia in synaptic and structural reorganization of hippocampal neurons [267]. Interestingly, it has also been shown that in status epilepticus (SE), where there is a prolonged seizure lasting longer than five minutes, ST8Sia IV-deficient mice demonstrate a reduced latency to the onset of kainite-induced SE as well as an increase in mortality [268]. The precise role that polySia has in "protection" following SE is still to be elucidated.

Huntington's disease

Huntington's disease (HD) is an autosomal dominant genetic disorder that is the result of an expanded number of CAG repeats within the huntingtin gene. Patients with this disease exhibit progressive motor defects, as well as cognitive dysfunction and dementia, ultimately resulting in death [269-271]. Work by van der Borght *et al.*

[272] demonstrated that in both wild-type and HD mouse models, the amount of polySia-NCAM within the hippocampus region of the brain decreases with age, however the reduction is much more profound in the HD model. However, the total number of hippocampal neurons between wild-type and HD mouse models were not significantly different and any stage during the HD disease process, suggesting that there is a loss of polySia-NCAM that is significant in HD. Since learning and cognitive training have previously shown to increase the amounts of polySia within the hippocampus [273, 274], these findings above correlate with a reduction in synaptic plasticity and the progressive loss of memory and cognition as the disease continues. Despite the significant reduction in polySia-NCAM, the precise mechanism behind this decrease, whether due to a loss of NCAM or a loss of polySia synthesis, still needs to be determined.

Cancer

Despite the necessity of polySia expression, both constitutively and transiently, within the adult mammal for ongoing synaptic plasticity and neuroprotection, aberrant expression of polySia is observed in a variety of human cancers [reviewed in 275]. Within neuronal tissue, polyST and polySia expression has been found to be increased in malignant astrocytomas, malignant gliomas, the neuroblastoma and a series of neuroendocrine tumors [276-280]. PolySia expression is mainly found in the more advanced stages of these cancers, where metastasis and a lower survival rate are common. As an example, autopsy samples of malignant astrocytomas demonstrate no staining for polySia in stages I and II, but exhibit a profound staining for polySia is stages III (anaplastic astrocytoma) and IV (Glioblastoma multiforme) [276]. The former of these states consist of slow growing, benign astrocytes. However, the latter stages consist of highly malignant cells covered in polySia that spread quickly to other parts of the brain, leading to the formation of multiple neurological defects. The average life expectancy of an individual diagnosed with stage III astrocytoma is 18 months, whereas an individual diagnosed with stage IV will typically survive 17 weeks without treatment, 30 weeks with radiation therapy, or surprisingly only 37 weeks with surgical removal of most of the tumor plus radiation therapy [281]. Interestingly, aberrant expression of polySTs and polySia has not only been found in tumors of neuronal tissue but also found expressed in tumors outside of the nervous system, including the small cell lung carcinoma, childhood rhabdomyosarcoma, and acute myeloid leukemia, as well as

colon, pancreatic and thyroid tumors [279, 282-287]. Within tumors, it is suggested that polySia interferes with NCAM properties by disrupting cell-cell adhesion [151] and altering cell signaling through growth factor receptors, thus promoting tumor invasiveness.

Polysialylation of synaptic cell adhesion molecule 1 and neuropilin-2

NCAM is by far the most abundantly polysialylated protein in mammalian systems. For this reason, NCAM has been the chosen protein to study the biological roles of polySia. From these studies, we have come to recognize polySia as a modification of NCAM that is necessary, critical, beneficial, but also detrimental, all at once. However, recent work has demonstrated an importance of polysialylation of two other polyST substrates, namely, synaptic cell adhesion molecule 1, and neuropilin-2 (Figure 6). These glycoproteins were investigated along with NCAM in the work presented in the following chapters and will be discussed below.

Synaptic cell adhesion molecule 1 (SynCAM 1) is an approximately 90 kDa, Ca^{2+}-independent cell surface adhesion molecule consisting of three immunoglobulin-like domains (Ig1-3) followed by a single-pass transmembrane region and a short cytosolic tail. SynCAM 1 is widely expressed throughout the brain within the synapses of both excitatory and inhibitory neurons where it forms homo- and heterophilic adhesive complexes and aids in the formation and maintenance of synapses [288]. SynCAM 1 has been found to be polysialylated on a single N-glycan in the Ig1 domain in a small subset of NG2 glial cells in the perinatal brain [289]. PolySia has been shown to reduce SynCAM 1-SynCAM 1 adhesion interactions *in vitro*, and is postulated to regulate the formation of a neuron-glial synapse [289]. Evidence also suggests that SynCAM 1 has roles in protein scaffolding and targeting through interaction with adaptor proteins via its intracellular PDZ domain interaction motif [290].

Neuropilin-2 (NRP-2) is a widely expressed transmembrane protein that plays roles in neuronal patterning, tumorigenesis and vascular development [reviewed in 291]. It is a 120-130 kDa protein consisting of a large extracellular region, followed by a single-pass transmembrane region and short cytoplasmic tail. Within its extracellular region, NRP-2 contains five distinct domains, including two complement C1r/C1s, Uegf, Bmp1 (CUB) domains, two coagulation factor 5/8 type domains, and one meprin/A5 protein/PTPmu (MAM) domain [292].

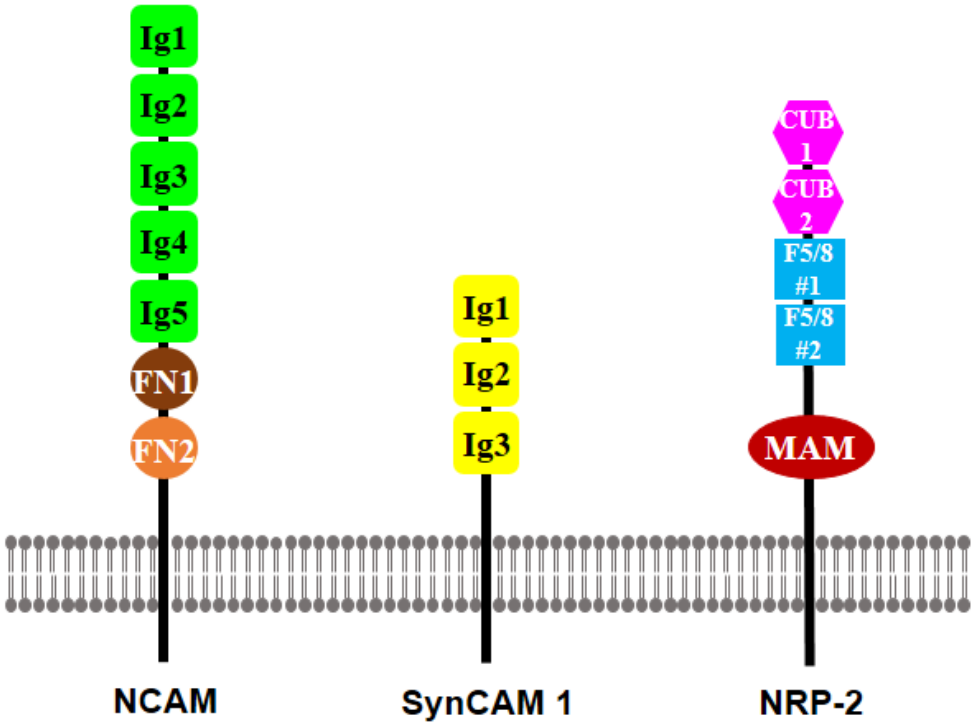

Figure 6. Depiction of NCAM, synaptic cell adhesion molecule 1 (SynCAM 1) and neuropilin-2 (NRP-2). NCAM, SynCAM 1, and NRP-2 are all cell surface polysialylated glycoproteins. NCAM is polysialylated on two asparagine residues in the Ig5 domain, whereas SynCAM 1 is polysialylated on a single N-glycan in the Ig1 domain, and NRP-2 is polysialylated on O-linked glycans.

Unlike the N-linked polysialylation of NCAM, NRP-2 has been shown to be polysialylated on O-linked glycans in mature dendritic cells [293]. Currently, the precise function of polySia on NRP-2 is still being debated, as it has been suggested to inhibit dendritic cell-induced T cell activation and proliferation [293], and conversely promote this process by enhancing dendritic cell migration to the lymph nodes through the stimulation of CCR7 receptor signaling [294]. A role for NRP-2 in tumor metastasis has been suggested by Gray *et al.* [295], who showed that inhibition of NRP-2 by shRNA in colorectal carcinoma cells reduces cell motility and invasiveness. This study is in line with evidence that neuropilin proteins have been shown to be present in a variety of human tumors, including those of the colon, prostate, kidney, bladder, stomach and pancreas [reviewed in 291, 296].

From the above studies, the biological significance of polySia modifying NCAM is well understood, and the significance of the polysialylation of SynCAM 1 and NRP-2 are beginning to take shape. However, what is truly lacking is an understanding of the precise biochemical mechanism by which the polySTs recognize and polysialylate NCAM, SynCAM 1 and NRP-2.

The human polysialyltransferases, ST8Sia II/STX and ST8Sia IV/PST

As previously mentioned, the two polySTs, ST8Sia IV/PST and ST8Sia II/STX, are responsible for catalyzing the synthesis of polySia on the termini of N-linked or O-linked glycans found on five known glycoproteins, as well as on their own N-glycans. PST and STX can act independently to polymerize polySia chains on glycans that are capable of extending near 57 residues within the brain and over 100 residues in human tumors.

Similar to all Golgi-associated glycosyltransferases, PST and STX are Golgi-localized type II membrane proteins with short N-terminal cytoplasmic tails, single pass transmembrane regions and large lumenal C-terminal regions [131, 297]. The cytoplasmic, transmembrane, and early portion of the C-terminal region (known as a stem region) are thought to confer proper localization and stability to the glycosyltranferases within the Golgi apparatus, while sequences in the later portion of the C-terminal region are responsible for catalytic activity [298]. To date, several regions within the sialyltransferases have been identified as having important roles in these enzymes' catalytic mechanism (Figure 7). For catalytic activity, four motifs have been identified. The *Large Sialyl Motif*

(SML) includes amino acid residues 141-185 in PST and 156-200 in STX. Current evidence suggests that residues within this motif are necessary for binding to the donor substrate, CMP-sialic acid [299]. The *Small Sialyl Motif (SMS)* includes amino acid residues 278-302 in PST and 293-317 in STX. This motif is important for binding to both the donor CMP-sialic acid and the acceptor glycan substrate [300]. The role of the *Very Small Sialyl Motif (VSMS)* is still unclear, however this region contains a histidine residue (His^{331} in PST, His^{346} in STX) that has been found to be conserved amongst all sialyltransferases, including the bacterial α2, 3/α2, 8-sialyltransferase CstII (His^{188}) [301]. Mutation of this residue to lysine abolishes the catalytic activity of PST [302]. *Motif III (M3)* is a sequence of 4 amino acid residues located between SMS and VSMS that is conserved in the sialyltransferases [303]. This region has been suggested to be important for recognition of the acceptor glycan substrate [304].

In addition to these catalytic motifs, two other regions outside the motifs have been proposed to be necessary for PST to effectively be able to add polysialic acid to target glycoproteins. The *Polybasic Region (PBR*) covers amino acids 71-105 in PST and 86-120 in STX. These sequences are found in polyST lumenal sequences prior to the start of the SML. Data suggest that sequences in this region are important for NCAM recognition [305]. The *Polysialyltransferase Domain (PSTD)* covers amino acids 246-277 in PST and 261-292 in STX. Similar to the PBR, the PSTD is basic in nature (pI is approximately 12). This region is thought to form electrostatic interactions with the negatively-charged growing polySia chain, thereby tethering the nascent chain to the enzyme and facilitating enzyme processivity [306]. In accordance with this, the polyanionic heparin molecule was able to inhibit polySia synthesis by PST.

Two other important features of the polySTs is the presence of disulfide bridges and protein autopolysialylation. Both polySTs contain two disulfide bridges that are formed between four cysteine residues that are conserved amongst the α2, 8-sialyltransferases. These disulfide bridges have been shown to be necessary for polyST activity, and are thought to allow for the proper positioning of the sialyl motifs relative to each other to allow for activity to occur [307]. Secondly, the polySTs are capable of adding polySia to their own N-glycans (autopolysialylation). STX contains six N-glycan residues, to which three are modified with polySia (Asn^{89}, Asn^{219}, Asn^{234}) [308]. PST has also been shown to contain five N-glycan asparagine residues, to which two are

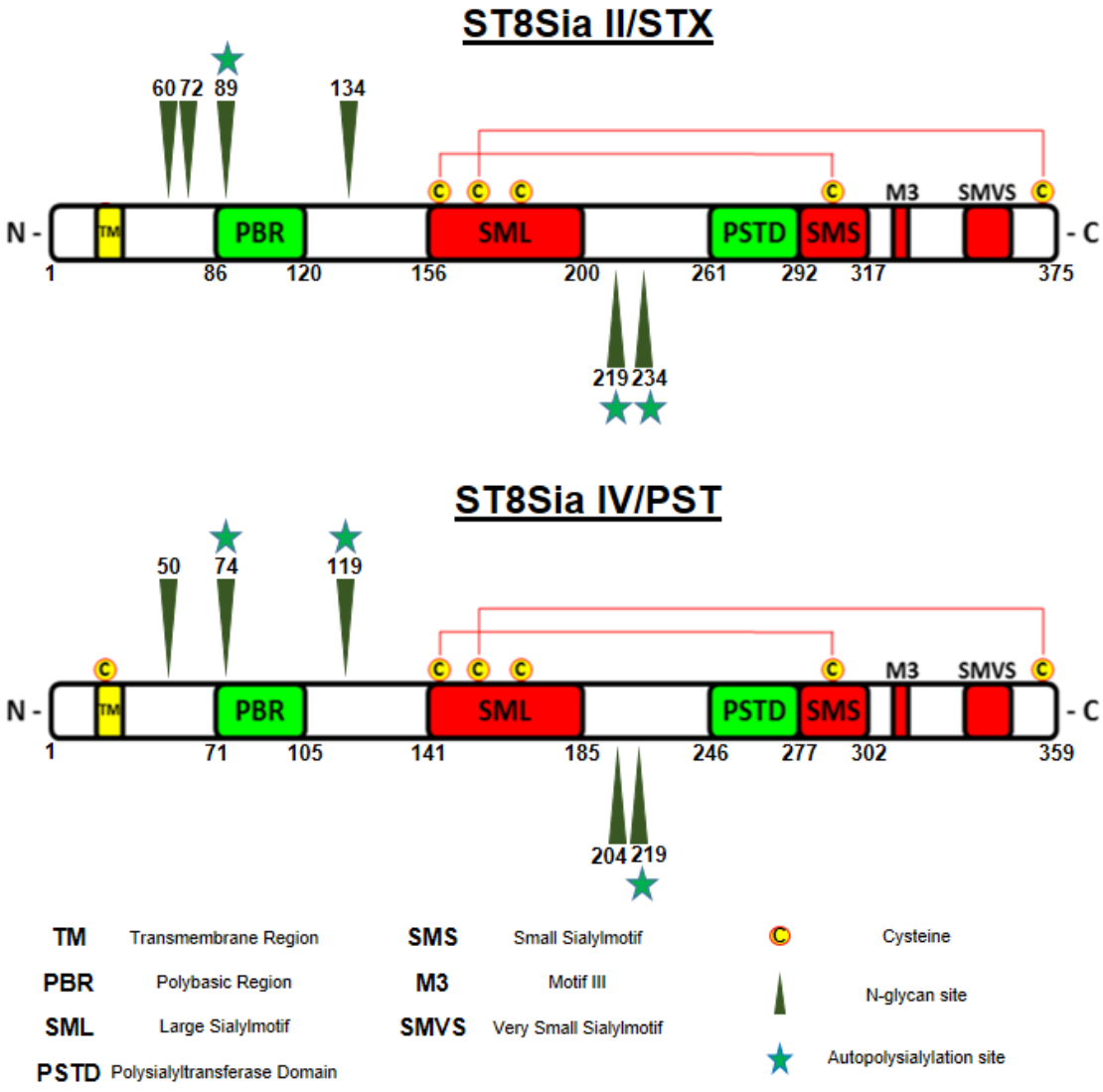

Figure 7. Schematic of the human polysialyltransferases: ST8Sia II (STX) and ST8Sia IV (PST). PST and STX contain the same catalytic sialylmotifs (*red*), transmembrane region, polybasic region, and polysialyltransferase domain. The 16 residue difference between these polySTs is due to a longer "stem" region in STX found between then TM and PBR regions. The sites of autopolysialylation were previously determined by Close *et al.* [308, 310].

modified with polySia [309, 310]. In the case of PST, Asn^{74} receives the majority of polySia, and Asn^{119} is also polysialylated [310]. In both cases however, mutating the sites of autopolysialylation did not prevent the resultant polyST mutant proteins from polysialylating NCAM, indicating that polyST autopolysialylation is not required for substrate polysialylation [308, 310].

Substrate polysialylation is a highly protein-specific process

The limited number of polyST substrates, the absence of any unique features on the modified core glycan structures, as well as data from polyST autopolysialylation and NCAM polysialylation studies detailed below, suggest that polysialylation is highly protein-specific and requires an initial protein-protein interaction between the polySTs and their substrates.

NCAM domain deletion and replacement studies

Although no structural information concerning the NCAM-polyST interaction has been obtained to date, traditional biochemical methods have given us important insight into regions within these proteins that may be required for their interaction. The extracellular portion of NCAM consists of five immunoglobulin-like domains (Ig1 – Ig5) and two fibronectin type III repeats (FN1 and FN2). Since N-linked polySia is added only to asparagine residues in the Ig5 domain, Close *et al.* [311] sought to determine the minimal NCAM structure necessary for polysialylation. Through deletion analysis, it was determined that an NCAM molecule consisting of only the Ig5 and FN1 domains was sufficient for polysialylation. Interestingly, deletion of the FN1 domain, replacement of FN1 with NCAM FN2, or the NCAM Ig5 domain alone, eliminated polysialylation of glycans on the Ig5 domain [311, 312], suggesting the NCAM FN1 domain is necessary for polySia addition to N-glycans on the Ig5 domain, and that it cannot be replaced with structurally similar fibronectin type II repeats. These studies confirmed earlier work by Nelson *et al.* [313], which suggested the necessity of the FN1 domain for polySia addition on Ig5, as replacement of NCAM FN1 with a fibronectin type III repeat of a related human L1 adhesion molecule ablated NCAM polysialylation. The necessity of FN1 is further suggested in an NCAM mutant consisting of only the FN1 and FN2 domains, without the Ig5 domain [311]. When expressed with PST, this protein was polysialylated on O-linked

glycans in the FN1 domain. Furthermore, co-immunoprecipitation analysis of NCAM domain deletion mutants found that any NCAM mutant that does not contain the NCAM FN1 domain did not bind to PST [314]. These data, collectively support a model whereby the FN1 domain serves as a recognition site for the polySTs, such that binding of the enzymes to NCAM at this location allows for the proper positioning of the enzyme to effectively add polySia to the designated N-glycans in the adjacent Ig5 domain [311].

NCAM structural studies

More recent studies have focused on testing the hypothesis that FN1 contains sequences that are required for polyST recognition. Mendiratta *et al.* [315] obtained the crystal structure of the FN1 domain of NCAM. Analysis of the structure demonstrated that there were two unique features present in NCAM FN1: (1) an α-helix consisting of 8 residues connecting the 4^{th} and 5^{th} β strands of the β sandwich that is not found in any other fibronectin type III repeats, and (2) a surface acidic patch formed by Asp^{497}, Asp^{511}, Glu^{512}, and Glu^{514}, which is not found in the NCAM FN2 domain. Replacement of the α-helix did not decrease NCAM polysialylation, but rather changed the location of polySia addition (O-linked glycans in the FN1 domain), suggesting that this α-helix was necessary for proper positioning of the polySTs, rather than in initial substrate recognition [315]. However, mutagenesis of acidic patch residues greatly reduced NCAM polysialylation [312]. To further investigate the role of the acidic patch in NCAM recognition by the polySTs, recent work in our lab has demonstrated that mutagenesis of acidic patch residues decreases but does not completely eliminate polyST binding, suggesting that the acidic patch is part of a larger recognition domain that may contain residues within the NCAM Ig5 domain [316]. In conjunction with this result, our laboratory also identified a second NCAM FN1 sequence ($Pro^{500}Ser^{501}Ser^{502}Pro^{503}$) that when mutated to alanines, reduces NCAM polysialylation and binding by PST [314]. This finding will be discussed later. A summary of the above mentioned NCAM polysialylation and binding studies can be found summarized in Tables II and III below.

TABLE II: POLYSIALYLATION ANALYSIS OF NCAM DELETION AND MUTAGENESIS MUTANTS

NCAM Mutant Created	**Polysialylation by PST?**
Ig1 – Ig2 – Ig3 – Ig4 – Ig5 – FN1 – FN2 (full-length wild-type NCAM)	**YES**
Ig5 – FN1	**YES**
Ig5	**NO**
Ig1 – Ig2 – Ig3 – Ig4 – Ig5 – FN2	**NO**
Ig1 – Ig2 – Ig3 – Ig4 – Ig5 – FN2 – FN2	**NO**
Ig1 – Ig2 – Ig3 – Ig4 – Ig5 – (LCAM)FN1 – FN2	**NO**
FN1 – FN2	**YES** (O-linked glycans in FN1)
NCAM D497A D511A E512A E514A (FN1 surface acidic patch mutant)	**DECREASED**
NCAM P500A S501A S502A P503A	**DECREASED**

TABLE III: CO-IMMUNOPRECIPITATION ANALYSIS OF SELECT NCAM MUTANT PROTEINS

NCAM Mutant Created	**Binds to PST?**
Ig1 – Ig2 – Ig3 – Ig4 – Ig5 – FN1 – FN2 (wild-type full-length NCAM)	**YES**
Ig5 – FN1 – FN2	**YES**
Ig5 – FN1	**YES**
Soluble FN1 – FN2	**YES**
Ig1 – Ig2 – Ig3 – Ig4 – Ig5 – FN2	**NO**
NCAM D497A D511A E512A E514A (FN1 surface acidic patch mutant)	**DECREASED**
NCAM P500A S501A S502A P503A	**DECREASED**

Since the Ig5 and FN1 domains are both required for effective NCAM polysialylation, Foley *et al.* [317] obtained the crystal structure of the NCAM Ig5-FN1 unit in an attempt to understand the structural relationship between these two domains. Upon completion of the structure, a series of N-glycosylation sites were engineered individually in the Ig5 domain of an NCAM molecule in which the 5th (ASN5) and 6th (ASN6) N-glycosylation sites were mutated in order to examine the flexibility of placement of sites for polysialylation in Ig5. These new N-glycosylation sites were engineered opposite the FN1 domain near the Ig4 domain, as well as near the 4th, 5th, and 6th N-glycosylation sites. Interestingly, it was found that a majority of these NCAM mutants were still capable of being polysialylated by PST, suggesting that there is flexibility in the placement of site for polysialylation in NCAM Ig5. However, whether the resultant polySia in these NCAM ASN5/ASN6 mutants was found on N- or O-glycans depended on the particular engineered N-glycosylation site created. When N-glycosylation sites that produced O-linked polysialylation in the NCAM mutant were introduced into wild-type NCAM, polysialylation returned to the 5th and 6th N-glycosylation sites. These results suggested that engineered N-glycosylation sites that led to O-linked polysialylation in an NCAM ASN5 and ASN6 mutant do not block access of the polyST to Ig5, but rather causes changes in surface structure that interferes with the ability of a polyST from properly engaging the Ig5 domain. Furthermore, these result suggest a potential role of NCAM Ig5 sequences in polyST recognition in addition to those from the NCAM FN1 domain. Further evidence of criteria within NCAM Ig5 that may mediate polyST recognition will be discussed a little later. These studies however do not detract from the fact that the NCAM FN1 domain and sequences within may serve as the primary recognition site for the polySTs. Even though O-linked polysialylation was found above in the placement of select glycosylation sites in Ig5, the fact that these sites were still glycosylated suggests that the polyST was still capable of engaging the NCAM molecule. Furthermore, previous results discussed earlier demonstrated that in the presence of only the FN1 and FN2 domains of NCAM, this protein is still polysialylated and can bind to PST [311, 314].

Analysis of NCAM and olfactory cell adhesion molecule FN1 domain sequences

Analysis of an acidic patch on the surface of the NCAM FN1 domain indicated that these residues were critical for NCAM binding and polysialylation, but that it may be part of a larger recognition complex [312]. To

examine the NCAM FN1 domain further for sequences that were critical for polyST recognition, our laboratory used homology modeling to compare the FN1 domain from NCAM to that of the olfactory cell adhesion molecule (OCAM) [318]. NCAM and OCAM contain the same extracellular domain structure of five Ig-like domains and two FN domains, are expressed at some of the same times and in some of the same places as NCAM, and their FN1 domains share approximately 37% sequence homology, but OCAM is not polysialylated [319, 320]. Comparison of the domain structure of NCAM and OCAM FN1 revealed two NCAM sequences that are significantly different: Pro^{510}-Tyr^{511}-Ser^{512} (PYS) and Gln^{516}-Val^{517}-Gln^{518} (QVQ). To examine the roles of these sequences in NCAM recognition, each was mutated individually to alanines in wild-type NCAM as well as an NCAM molecule consisting of only the cytosolic tail, transmembrane region, and FN1 and FN2 domains (NCAM7) (summarized in Table IV). Replacement of PYS slightly reduced NCAM polysialylation and practically eliminated NCAM7 polysialylation, suggesting a role for PYS in NCAM recognition similar to that of the NCAM FN1 acidic patch. In contrast, replacement of QVQ in NCAM shifted polysialylation to O-glycans, similar to replacement of the FN1 α-helix, suggesting that it has a role in polyST positioning. Additionally, in NCAM7, QVQ replacement did not eliminate O-linked glycan polylsialylation, but shifted to a lower molecular mass, suggesting that QVQ might have some impact on the efficiency of NCAM recognition.

Are the PYS and QVQ sequences sufficient for NCAM recognition and polysialylation? To better understand this question, an NCAM chimera was created in which the NCAM FN1 domain was replaced with OCAM FN1 [318] (summarized in Table V). Surprisingly, the resultant chimera was still polysialylated, albeit to a lesser extent, suggesting that the NCAM FN1 is more effective in promoting recognition than OCAM FN1. Furthermore, insertion of a three amino acid linker (Ala-Leu-Asp or Ala-Ala-Ala) between the OCAM FN1 and NCAM Ig5 domains significantly reduced Ig5 polysialylation, indicating that a precise relationship between Ig5 and FN1 must be maintained in order for effective polysialylation to occur. But more importantly, replacing different combinations of QVQ, PYS, FN1 acidic patch or α-helix into the NCAM-OCAM FN1 chimera indicated that PYS is likely to maintain the FN1 domain in a conformation that promotes the generation of sialylated O-

glycans, whereas the acidic patch is primarily responsible for the polysialylation of the NCAM-OCAM FN1 chimera.

TABLE IV: COMPARISON OF THE EFFECTS OF NCAM FN1 PYS AND QVQ MUTATIONS ON THE POLYSIALYLATION OF NCAM AND NCAM7 BY ST8SIA IV/PST

Mutation	**NCAM Polysialylation?**	**NCAM7 Polysialylation?** (O-linked polySia)
No NCAM/NCAM7 mutation	**YES**	**YES**
QVQ → AAA	**YES** (Switched to O-linked)	**YES** (Lower molecular weight polySia)
PYS → AAA	**SLIGHT DECREASE**	**NO**

TABLE V: COMPARISON OF THE POLYSIALYLATION OF NCAM, OCAM, AND AN NCAM-OCAM FN1 CHIMERA BY ST8SIA IV/PST

Substrate	**Ig5 N-glycan Polysialylation by PST?**
NCAM	**YES**
OCAM	**NO**
NCAM-OCAM FN1	**50% as much polySia as NCAM**
NCAM-OCAM FN1 + ALD linker	**NO**
NCAM-OCAM FN1 + AAA linker	**NO**

In contrast to the above studies from Foley *et al.*, Thompson *et al.* [314] examined sequences that were found to be similar between the FN1 domains of NCAM and OCAM and identified three sequences near the NCAM Ig5/FN1 interface: $Pro^{500}Ser^{501}Ser^{502}Pro^{503}$ (PSSP), $Gly^{526}Gly^{527}Val^{528}Pro^{529}Ile^{530}$ (GGVPI), and $Asn^{580}Gly^{581}Lys^{582}Gly^{583}$ (NGKG). Upon examination of these three sequences, it was found that when PSSP was mutated to alanines, NCAM and NCAM7 polysialylation were significantly reduced, as was polyST-NCAM binding [314]. These results are once again in line with studies on the acidic patch, suggesting a role of PSSP in recognition. In contrast, GGVPI and NGKG reduced NCAM polysialylation similarly to PSSP, but did not substantially affect NCAM7 polysialylation, suggesting that these sequences at the interface of Ig5 and FN1 are critical for stabilizing the Ig5-FN1 linker region and thus promoting the proper positioning of the two domains to allow for proper polysialylation of full-length NCAM.

In sum thus far, NCAM studies have indicated that the NCAM FN1 domain is necessary for effective polysialylation at the neighboring Ig5 domain as it may contain a recognition surface for the polySTs. This fact is demonstrated by the fact that any NCAM construct containing the FN1 domain, whether it contains the Ig5 domain or not, as in the case of NCAM7, will bind to a polyST and will be polysialylated. Furthermore, sequences within the FN1 domain have been shown to be important for effective NCAM recognition, including a surface acidic patch and PSSP, which may be part of a larger recognition interface on NCAM FN1.

NCAM Ig5 domain studies

In an attempt to understand why OCAM is not polysialylated even though the OCAM FN1 can partially replace NCAM FN1 and promote polysialylation of an NCAM-OCAM FN1 chimera, Thompson *et al.* [316] compared the NCAM and OCAM Ig5 domains for features that might explain why OCAM is not polysialylated. To determine the significance of the Ig5 domain as a whole, three OCAM chimeras were created in which either the Ig5 or FN1 domain, or both, were replaced with the corresponding domains from NCAM (see Table VI). As expected, wild-type OCAM and OCAM with the NCAM FN1 domain only were not polysialylated. However, OCAM with the NCAM Ig5 domain or with the NCAM Ig5/FN1 tandem demonstrated an increase in polysialylation of 19% and 55%, respectively, cumulatively indicating that the OCAM FN1 domain is weakly

recognized by the polySTs and that the OCAM Ig5 domain may be prohibiting OCAM polysialylation. A search for residues that were significantly different between OCAM and NCAM Ig5 found two residues near OCAM's 6th (ASN6) and 7th (ASN7) N-glycosylation sites (equivalent to the 5th and 6th N-glycosylation sites in NCAM). Namely, these residues were OCAM Lys^{444} (adjacent to Asn^{445}, which is ASN6 for OCAM) and OCAM Arg^{472} (two residues away from Asn^{474}, which is ASN7 for OCAM), whose equivalent positions in NCAM are filled by Ser^{448} and Asn^{476}, respectively. Replacement of Ser^{448} with Lys and Asn^{476} with Arg, significantly reduced or eliminated NCAM polysialylation as well as polyST binding, suggesting that these large basic residues in OCAM Ig5 prevent its polysialylation (Table VII). Furthermore, replacement of these residues in NCAM with alanines or glutamic acid significantly reduced polysialylation and partially reduced binding, suggesting that Ser^{448} and Asn^{476} are part of a larger recognition surface in the NCAM Ig5 domain (Table VII).

TABLE VI: COMPARISON OF THE POLYSIALYLATION OF OCAM-NCAM CHIMERAS BY ST8SIA IV/PST

Substrate	**Polysialylation by PST?**
OCAM	**NO**
OCAM-NCAM FN1	**NO**
OCAM-NCAM Ig5	**19% increase in polySia over OCAM**
OCAM-NCAM Ig5-FN1	**55% increase in polySia over OCAM**
OCAM-NCAM Ig5 + NCAM FN1 acidic patch	**66% increase in polySia over OCAM**

TABLE VII: EFFECTS OF LARGE OCAM IG5 BASIC RESIDUES ON NCAM POLYSIALYLATION AND BINDING BY ST8SIA IV/PST

Substrate	**Polysialylation by PST?** (Percent compared to NCAM)	**Binding by PST?** (Percent compared to NCAM)
NCAM	**100%**	**100%**
NCAM S448K	**15%**	**52%**
NCAM N476R	**3%**	**55%**
NCAM S448K / N476R	**3%**	**26%**
NCAM S448A / N476A	**34%**	**79%**
NCAM S448E / N476E	**37%**	**84%**

Since it has been previously shown that the NCAM FN1 domain is critical for polyST recognition and subsequent polysialylation of NCAM, the finding that sequences in NCAM Ig5 may also be important for recognition raised the question as to which domain and residues are most critical for NCAM recognition. Two findings suggest that the NCAM FN1 domain is the primary recognition site [316]. First, mutation of the 5th and 6th N-glycosylation sites in NCAM Ig5 did not alter polyST binding, suggesting that the NCAM ig5 N-glycans do not play a substantial role in NCAM-polyST interaction. Secondly, and more importantly, creation of the NCAM FN1 surface acidic patch within the OCAM FN1 domain by simply adding two acidic residues significantly increased the polysialylation of an OCAM-NCAM Ig5 chimera, suggesting that the NCAM FN1 and the acidic patch within play a key role in the recognition of NCAM by the polySTs (Table VI).

Polysialyltransferase studies

The identification of sequences in NCAM FN1 that serve for enzyme recognition suggests that there are complimentary sequences in the polySTs that are responsible for NCAM recognition. To try to pinpoint regions within the polySTs that were necessary for NCAM polysialylation by the polySTs, Angata *et al.* [321] evaluated the requirements for PST activity and NCAM polysialylation using chimeric enzymes in which portions of a soluble PST enzyme (residues 40-359) were removed and replaced with the corresponding region from a related α-2,8-sialyltransferase, ST8Sia III. ST8Sia III is capable of enzyme autopolysialylation (similar to PST and STX), but is unable to polysialylate NCAM [322]. Additionally, ST8Sia III shares 34.8% sequence identity with PST, most of which is found within the catalytic sialylmotifs. Analysis of these chimeras indicated that distinct regions within the polySTs were responsible for NCAM recognition and NCAM polysialylation, and it was proposed that two regions outside the sialylmotifs were necessary for NCAM polysialylation by the PST: (1) amino acids 40-127 (prior to SML), and (2) 194-267 (between SML and SMS). Further data has suggested that the former region may serve an NCAM recognition function [305], whereas the latter region may be involved in protecting the fidelity and enhancing the processivity of polysialylation by forming interactions with a nascent growing chain [306]. In terms of the first region, we found that replacement of specific residues in the PBR of PST (amino acids 71-105) significantly reduced NCAM polysialylation, while autopolysialylation was not affected, indicating that residues within the PBR, which is outside of the catalytic sialylmotifs, are important for proper NCAM polysialylation and hinting at a loss of NCAM recognition by PST since catalytic activity was not lost [305]. Despite the above studies, these regions in PST, and potentially the corresponding regions in STX, remain very broad, and more specific criteria within these regions that are required for NCAM polysialylation have not yet been identified.

Current working hypothesis of substrate polysialylation

The above data indicate that the NCAM FN1 region, which itself is not polysialylated, is absolutely necessary for the polysialylation of NCAM at the Ig5 domain. Furthermore, the PST PBR, which is not within the catalytic portion of the enzyme, is also necessary for proper NCAM poylsialylation. The necessity of the NCAM FN1 domain and the polyST PBR region for NCAM polysialylation is consistent with the view that the interaction

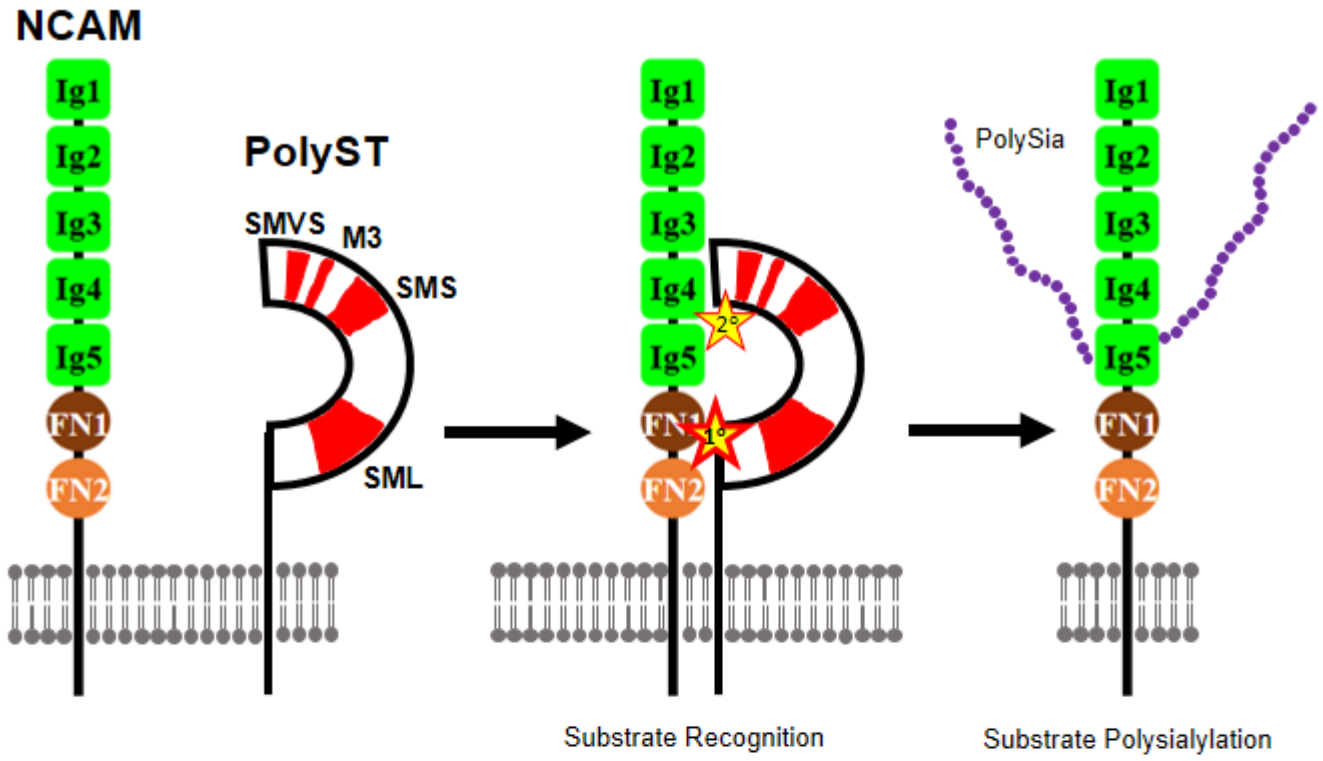

Figure 8. Current working model of substrate polysialylation by the polySTs. The working model of substrate polysialylation is based on data obtained from NCAM and PST studies. From these studies, it is currently theorized that the polySTs and NCAM form a critical protein-protein interaction utilizing sequences within the NCAM FN1 domain (*1°, yellow star*). This interaction serves to stabilize the polyST next to NCAM. Recent data from our laboratory further suggest that secondary contacts between the polyST and NCAM occur utilizing residues within the NCAM Ig5 domain (*2°, yellow star*), and this interaction may serve to properly position the polyST with NCAM Ig5 N-glycans. Following these interactions, polysialylation occurs on the 5th and 6th N-glycosylation sites on the NCAM Ig5 domain.

between the polySTs and NCAM is highly protein-specific and requires a necessary initial recognition interaction involving FN1 and residues within the polyST PBR, conferring protein specificity (Figure 8). More recent results suggest that a secondary interaction between the polySTs and the NCAM Ig5 domain is important for proper positioning of the polyST and Ig5 N-glycans for polysialylation [316]. However, this interaction is not sufficient to mediate the protein specificity and NCAM polysialylation.

Polysialic acid as a potential pharmaceutical target in cancer

PolySia has been found in the late stages of numerous aggressive human cancers where it has been shown to promote the metastatic potential and invasiveness of tumors [reviewed in 323]. Furthermore, using Endo N to remove polySia from glioblastoma multiforme cancer cells reduces the migration and invasion of multiple tumors [323, 324]. With this in mind, the questions becomes can we target polySia as a potential therapeutic in cancer treatments? While there is not enough evidence at the current time to decide if polySia can be targeted, some studies indicate that it could definitely be a possibility. The reason that it is difficult to establish a vaccine against polySia is that our immune systems have developed tolerance to this glycan modification. This tolerance is a result of the extremely high levels of polySia found in the developing embryo. Despite this, Krug *et al.* [325] found that by genetically engineering N-propionylated-PolySia (NP-PolySia) on the surfaces of mouse leukemia cells, the cells' growth and metastasis could be slightly attenuated following immunotherapy with an antibody against NP-PolySia. Of even more clinical relevance was a clinical trial by Krug *et al.* [325] in which small cell lung carcinoma patients were treated with a vaccine made from wild-type polySia or from NP-PolySia. All but one patient who was vaccinated with NP-PolySia developed IgM antibodies that were able to cross-react with endogenous polySia on small cell lung carcinoma cells. On the contrary, only one patient vaccinated with the wild-type polySia vaccine developed an IgM response, suggesting that NP-PolySia could be used as a therapeutic agent against cancer cell polySia. Issues such as cross-reactivity in regions where there is beneficial ongoing polySia production, as well as penetration of the blood-brain barrier, must still be investigated. It should be noted as well that NP-PolySia has thus far only slightly attenuated the metastatic spread of cancer cells, thus more investigations into other potential therapeutics are warranted.

Significance

The modification of NCAM with polySia has been shown to be both necessary (brain development, neural regeneration) and detrimental (cancer cell metastasis) to humans, making substrate polysialylation an important process that needs to be fully understood. Currently, the mechanism by which the polySTs are able to effectively interact with and polysialylate substrates is not yet fully understood. A greater understanding of the specific regions and residues within the polySTs and NCAM that regulate their interaction is necessary before we can potentially develop therapeutic agents that could target the interaction to block substrate polysialylation. The overall goal of the work presented here is to elucidate the mechanism by which the polySTs polysialylate their substrates. Specifically, this work aims to identify and analyze more specific regions and residues outside the catalytic sialylmotifs of PST and STX that are necessary for recognition and resultant polysialylation of NCAM, SynCAM 1 and NRP-2. I began my thesis work with a focus on identifying a region within PST that is critical for the recognition of NCAM. Using these results, I then expanded my findings to include a more thorough analysis of residues within both PST and STX that are critical for interaction with NCAM, SynCAM 1 and NRP-2.

CHAPTER II

MATERIALS AND METHODS

Acquisition of materials and reagents

The cDNA for full-length human NCAM140 and the SW2 small cell lung carcinoma cell line were gifts from Dr. Nancy Kedersha (Brigham and Women's Hospital, Boston, MA). The cDNAs for full-length human neuropilin-2 and synaptic cell adhesion molecule 1 were obtained from Dr. Nicholas Stamatos (University of Maryland School of Medicine, Baltimore, MD) and Dr. Thomas Biederer (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT), respectively. The cDNAs for full-length human ST8Sia IV/PST and ST8Sia II/STX were obtained from Dr. Minoru Fukuda (Sanford Burnham Medical Research Institute, La Jolla, CA) and Dr. John Lowe (Genentech, South San Francisco, CA), respectively.

Tissue culture media and reagents, including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), OPTI-MEM I, Lipofectin, Lipofectamine, and Lipofectamine 2000, as well as all oligonucleotides, mouse monoclonal anti-Myc epitope tag antibody for immunocytochemistry, anti-V5 epitope tag antibody, and 4', 6-diamidino-2-phenyl-inidole, dihydrochloride (DAPI) were purchased from Invitrogen (Carlsbad, CA). The QuikChange™ site-directed mutagenesis kit and *Pfu* DNA polymerase were purchased from Stratagene (La Jolla, CA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). All plasmid DNA purification kits were obtained from Qiagen (Valencia, CA). Protease inhibitors were purchased from Roche Applied Science (Indianapolis, IN). Poly-L-lysine microscope coverslips and rat anti-mouse CD56 (12F8) antibody were purchased from BD Biosciences (San Jose, CA). Protein A-Sepharose beads were purchased from GE Healthcare (Piscataway, NJ). Mouse monoclonal anti-Myc epitope tag antibody for immunoblotting was obtained from Cell Signaling Technologies (Danvers, MA). Rabbit polyclonal anti-Myc epitope tag antibody for immunoprecipitation was purchased from Abcam (Cambridge, MA). Horseradish peroxidase (HRP)-, Fluorescein isothiocyanate (FITC)-, and Rhodamine (TRITC)-conjugated goat anti-mouse and goat anti-rat secondary antibodies, were obtained from Jackson ImmunoResearch (West Grove, PA). Precision Plus™ Protein standards,

as well as 4-15% Mini-PROTEAN TGX precast gels, were purchased from Bio-Rad (Richmond, CA). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH). SuperSignal West Pico chemiluminescence reagent was obtained from Pierce (Rockford, IL). Blue Ultra Autorad film was purchased from BioExpress (Kaysville, UT). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Hanover Park, IL).

Creation of membrane-associated truncated PST mutants

The ST8Sia IV/PST cDNA was cloned into the EcoRV and XbaI sites of a previously digested pcDNA3.1/Myc-HisB mammalian expression vector containing a carboxyl terminal Myc epitope tag, a stop codon prior to the His_6 tag, and an ampicillin resistance gene outside of the cloning site. The resulting plasmid containing the DNA sequence for PST-Myc served as the template for the creation of all truncated PST mutants.

Step 1: Insertion of XbaI restriction site into PST cDNA

The first step in creating PST mutants that are truncated from the carboxyl terminus is the insertion of an XbaI restriction site (5'-TCTAGA-3') into the PST cDNA immediately downstream of the codon corresponding to the amino acid that will serve as the truncated carboxyl terminal residue of PST. The XbaI sites were inserted into the PST cDNA using the QuikChange™ site-directed mutagenesis kit and *Pfu* DNA polymerase according to manufacturer's instructions, utilizing the oligonucleotide primers listed in Table VIII. All mutagenesis reactions were prepared as follows: 5 µl 10X reaction buffer, 50 ng of DNA template, 125 ng of each appropriate oligonucleotide primer, 1 µl of 10 mM dNTP mix (2.5 mM of each dNTP), 1 µl *Pfu* DNA polymerase (2.5 units), and dH_2O up to a final volume of 50 µl. The reaction cycling conditions were as follows: denaturation at 95°C for 30 seconds / 18 cycles of 95°C for 30 seconds, 55°C for one minute, 68°C for 13 minutes / 68°C for 5 minutes. Following reaction cycling, 1 µl (20 units) of DpnI restriction enzyme was added to each mutagenesis reaction and the mixtures were incubated for one hour at 37°C in order to digest the parental DNA (i.e., methylated and hemi-methylated, non-mutated dsDNA).

Step 2: Plasmid DNA purification and sequencing

Fifty microliter aliquots of *E. coli* XL1-Blue competent cells were transformed with 1 µl of each DpnI-digested mutagenesis reaction by heat-shocking the bacteria for 45 seconds at 42°C, followed by recovery for three minutes at 4°C. After recovery, 500 µl of LB media was added to each aliquot of transformed bacteria and the bacteria was grown at 37°C for three hours, then streaked on an LB agar plate containing 100 µg/mL ampicillin (LB-amp) and incubated at 37°C for 18 hours. Bacteria colonies that formed were then picked from the plate with a sterile wooden stick, placed into conical tubes containing 3 mL of LB-amp media and grown for 18 hours at 37°C with shaking at 240 rpm. The following day, the plasmid DNA was purified from the bacteria using the Qiagen QIAprep Spin Miniprep Kit utilizing the manufacturer's instructions. Additionally, 10 µl of the grown bacteria was spotted on an LB-amp agar plate prior to purification for the next step in the protocol. The purified plasmid DNA was then checked for accuracy and the desired mutation by DNA sequencing performed by the DNA Sequencing Facility at the Research Resources Center at the University of Illinois at Chicago.

TABLE VIII: PRIMERS USED TO CREATE MYC-TAGGED TRUNCATED ST8SIA IV/PST PROTEINS

Truncated ST8Sia IV/PST	**Template**	**Oligonucleotide Primers**
PST140	**PST-Myc**	5'-CGCAGGTTTAAGACTCTAGACTGTGCAGTTG-3' 5'-CAACTGCACAGTCTAGAGTCTTAAACCTGCG-3'
PST127	**PST-Myc**	5'-CATGATCTACATAGCCTCCTTCTAGAAGTTTCACCAATG-3' 5'-CATTGGTGAAACTTCTAGAAGGAGGCTATGTAGATCATG-3'
PST71	**PST-Myc**	5'-GTAGAAGGTTGGAATCTAGATTCCTCTTTG-3' 5'-CAAAGAGGAATCTAGATTCCAACCTTCTAC-3'
PST62	**PST-Myc**	5'-GCTGGCTCTTCAATTCTAGAGCACAATGTAGAA-3' 5'-TTCTACATTGTGCTCTAGAATTGAAGAGCCAGC-3'

Step 3: Digestion of PST cDNA with XbaI restriction enzyme

The PST cDNA was previously cloned into the pcDNA3.1/Myc-HisB vector such that an XbaI restriction site exists immediately downstream of the full-length PST cDNA but prior to the start of the Myc epitope tag. Engineering a second XbaI site immediately downstream of the codon that corresponds to the amino acid that will serve as the new carboxyl terminal of PST, as performed in Step 1 above, allows us to use the XbaI restriction enzyme to remove the undesired PST cDNA sequence while keeping the desired truncated PST sequence in the vector with the Myc epitope tag remaining at the carboxyl terminal.

The mutated PST cDNA from Step 2 was purified from *E. coli* XL1-Blue competent cells with the DNA plasmid purification method described above, utilizing the bacteria previously spotted on an LB-amp plate. Following purification, the DNA was incubated with XbaI restriction enzyme for 18 hours at 37°C. The restriction enzyme digestions were prepared as follows: 3 µl 10X reaction buffer, 2 µg of template plasmid DNA, 3 µl XbaI restriction enzyme (60 units), and dH_2O up to a final reaction volume of 20 µl.

Step 4: Purification of digested plasmid DNA and ligation

Following XbaI digestion, reaction mixtures were run on a 1.6% agarose gel and the cleaved vector was recovered and purified directly from the gel using the Qiagen QIAquick Gel Extraction Kit according to the manufacturer's instructions. The purified DNA consists of linear double-stranded DNA containing an XbaI site on both ends separating the desired truncated PST cDNA sequence and the Myc epitope tag. To unite these two entities, the DNA was incubated with T4 DNA ligase for three hours at 16°C. The ligation reactions were prepared as follows: 2 µl 10X T4 DNA ligase buffer, 4 µl linearized DNA, 1 µl T4 DNA ligase (400 units), and dH_2O up to a final volume of 20 µl. To confirm that the correct DNA sequences are present, XL1-Blue cells were transformed with the entire ligation mixture, and the DNA was purified and sent for sequencing as described above.

Step 5: Large-scale purification of truncated PST-Myc cDNA

For each plasmid DNA, a 400 µl colony of LB-amp was inoculated with the appropriate bacterial colony from the plate spotted prior to DNA purification from above. This colony was grown for 18 hours at 37°C with

shaking at 240 rpm. The bacteria were harvested by centrifugation at 6,000 rpm, and purified DNA was obtained using the Qiagen Plasmid Maxi Kit according to the manufacturer's instructions. The resultant DNA pellet was resuspended in 600 µl of dH_2O and the concentration determined by measuring absorbance at 260 nm.

Construction of soluble PST-Myc and soluble PST140-Myc mutants

Soluble PST (sPST-Myc), consisting of a full-length PST protein lacking the cytosolic tail and transmembrane region, was generated in two steps as described previously [314]. Briefly, canine pre-pro-insulin signal peptide was amplified from a previously generated soluble NCAM-V5 cDNA construct using primers 1 and 2 in Table IX. The resultant DNA was digested with Hind III and EcoRV restriction enzymes and ligated into the pcDNA3.1/Myc-HisB vector using the method described above, creating a vector with a secretory pathway signal peptide followed by a Myc epitope tag. The cDNA coding sequences for the ST8Sia IV/PST cytosolic tail and transmembrane region were then excised from the wild type PST using primers 3 and 4 in Table IX. The resulting PST cDNA was digested with EcoRV and XbaI, and then ligated into the modified signal peptide/Myc tag vector. The final cDNA for sPST-Myc contains an N-terminal secretory pathway signal peptide followed by the Golgi-lumenal sequence of PST and a Myc epitope tag. Soluble PST140 (sPST140-Myc), consisting of the Golgi-lumenal residues of PST up to residue 140, was generated from this vector using mutagenesis with primers listed in Table IX, followed by XbaI digestion and ligation as described above.

Creation of PST-Myc H331K, STX-Myc H346K, and all PST/STX PBR point mutants

The ST8Sia II/STX cDNA was cloned into the EcoRV and XbaI sites of a previously digested pcDNA3.1/Myc-HisB mammalian expression vector containing a carboxyl terminal Myc epitope tag, a stop codon prior to the His_6 tag, and an ampicillin resistance gene outside of the cloning site. All PST-Myc PBR point mutations were previously created by Foley *et al.* [305] in the laboratory. STX-Myc PBR point mutations were created utilizing the oligonucleotide primers listed in Table X. Catalytically-inactive full-length PST-Myc H331K, STX-Myc H346K, and associated PBR point mutants, were created using the oligonucleotide primers listed in Table XI.

Each of these mutant proteins were created using site-directed mutagenesis, purified, and sequenced as described above.

TABLE IX: PRIMERS USED TO CREATE SOLUBLE MYC-TAGGED ST8SIA IV/PST PROTEINS

Soluble ST8Sia IV/PST	**Template**	**Oligonucleotide Primers**
sPST (#1,2)	**sNCAM-V5**	5'-AAAAAAAAGCTTGCTAGCTTGCTTGTTCTTTTTGCAG-3' 5'-AAAAAAGATATCAGAGTCAACGAAGGCTGCGGTG-3'
sPST (#3,4)	**PST-Myc**	5'-AAAAAAGATATCTCAATCTTCCAGCACAATGTAGAAGG-3' 5'-AAAAAATCTAGACCTTGCTTTACACACTTTCCTGTTGTC-3'
sPST140	**sPST-Myc**	5'-CGCAGGTTTAAGACTCTAGACTGTGCAGTTG -3' 5'-CAACTGCACAGTCTAGAGTCTTAAACCTGCG -3'

TABLE X: PRIMERS USED TO CREATE MYC-TAGGED ST8SIA II/STX PBR MUTANT PROTEINS

ST8Sia II/STX PBR Mutant	**Template**	**Oligonucleotide Primers**
STX R87A	**STX-Myc**	5’-GCCTCGTCCAAATGCGCACATAACCAGACGCTC-3’ 5’-GAGCGTCTGGTTATGTGCGCATTTGGACGAGGC-3’
STX R95A	**STX-Myc**	5’-CAGACGCTCTCTCTGGCGATCAGGAAGCAGATT-3’ 5’-AATCTGCTTCCTGATCGCCAGAGAGAGCGTCTG-3’
STX R97A	**STX-Myc**	5’-CTCTCTCTGAGGATCGCGAAGCAGATTTTAAAG-3’ 5’-CTTTAAAATCTGCTTCGCGATCCTCAGAGAGAG-3’
STX K98A	**STX-Myc**	5’-CTGAGGATCAGGGCGCAGATTTTAAAGTTC-3’ 5’-GAACTTTAAAATCTGCGCCCTGATCCTCAG-3’
STX K102A	**STX-Myc**	5’-AGGAAGCAGATTTTAGCGTTCTTGGATGCTGAA-3’ 5’-TTCAGCATCCAAGAACGCTAAAATCTGCTTCCT-3’
STX K108A	**STX-Myc**	5’-CTTGGATGCTGAAGCGGACATTTCTGTC-3’ 5’-GACAGAAATGTCCGCTTCAGCATCCAAG-3’
STX K114A	**STX-Myc**	5’-GACATTTCTGTCCTAGCGGGAACCCTGAAGCCT-3’ 5’-AGGCTTCAGGGTTCCCGCTAGGACAGAAATGTC-3’
STX K118A	**STX-Myc**	5’-CTAAAGGGAACCCTGGCGCCTGGAGATATTATT-3’ 5’-AATAATATCTCCAGGCGCCAGGGTTCCCTTTAG-3’

TABLE XI: PRIMERS USED TO CREATE PST H331K, STX H346K AND ASSOCIATED PBR MUTANTS

ST8Sia IV/PST Mutant	Template (Myc-tagged)	Oligonucleotide Primers
PST H331K	**PST**	5'-CCAATGCAAGCCCTAAGAGAATGCCATTAG-3' 5'-CTAATGGCATTCTCTTAGGGCTTGCATTGG-3'
PST H331K R72A	**PST R72A**	
PST H331K R82A	**PST R82A**	
PST H331K K83A	**PST K83A**	
PST H331K R87A	**PST R87A**	
PST H331K R93A	**PST R93A**	
PST H331K K99A	**PST K99A**	
PST H331K K103A	**PST K103A**	
ST8Sia II/STX Mutant	**Template (Myc-tagged)**	**Oligonucleotide Primers**
STX H346K	**STX**	5'-CAGGCCAGCCCGAAGACCATGCCCTTG-3' 5'-CAAGGGCATGGTCTTCGGGCTGGCCTG-3'
STX H346K R87A	**STX R87A**	
STX H346K R95A	**STX R95A**	
STX H346K R97A	**STX R97A**	
STX H346K K98A	**STX K98A**	
STX H346K K102A	**STX K102A**	
STX H346K K108A	**STX K108A**	
STX H346K K114A	**STX K114A**	
STX H346K K118A	**STX K118A**	

Immunofluorescence analysis of wild-type and mutant PST and STX enzyme localization and activity

Transfection of COS-1 cells on coverslips

COS-1 cells maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS) were plated onto 12-mm glass coverslips and grown in a 37°C, 5% CO_2 cell incubator until 50-70% confluent. Cells on each coverslip were then transfected with 0.5 µg of Myc-tagged PST, sPST, mutant PST, STX or mutant STX cDNA in 300 µl of OPTI-MEM I supplemented with 3 µl of Lipofectin transfection reagent. Cells were then incubated with the transfection mixture for six hours at 37°C, followed by the addition of one milliliter of DMEM, 10% FBS, and allowed to grow in the incubator for 18 hours.

Immunofluorescence staining of COS-1 cells

Post-transfection, cells were washed with phosphate-buffered saline (PBS) then permeabilized with -20°C methanol to view internal structures as well as the cell membrane. Following two more washes with PBS, the cells were blocked in immunofluorescence blocking buffer (5% normal goat serum in PBS) for one hour, then incubated at room temperature for two hours with the following primary antibodies diluted in immunofluorescence blocking buffer: anti-Myc epitope tag antibody (1:250 dilution) to detect expression and localization of the polyST proteins, and OL.28 anti-polySia antibody (1:100 dilution) to determine polyST catalytic activity by detecting protein autopolysialylation. After incubation, the cells were washed twice with PBS, then incubated for one hour with the following secondary antibodies diluted in blocking buffer: FITC-conjugated goat anti-mouse IgG (1:100 dilution) to visualize protein localization, and TRITC-conjugated goat anti-mouse IgM (1:100 dilution) to visualize autopolysialylation. After washing four times with PBS, the cells were treated for five minutes with 300 nM DAPI diluted in PBS. Following a final wash with PBS, the coverslips were then mounted on glass microscope slides using 20 µl mounting medium (15% Vinol 205 polyvinyl alcohol (w:v), 33% glycerol (v:v), 0.1% azide, pH 8.5). Cells were visualized with a Zeiss Axiovert 200 M inverted confocal microscope using a 63X oil immersion objective.

Analysis of sPST and sPST140 secretion from COS-1 cells

Transfection of COS-1 cells on tissue culture plates

COS-1 cells maintained in DMEM media, supplemented with 10% FBS, were plated onto 100-mm tissue culture plates and grown in a 37°C, 5% CO_2 incubator until 50-70% confluent. Cells were transfected with 20 µg of Myc-tagged sPST or sPST140 cDNA in 3 mL OPTI-MEM I containing 30 µl Lipofectin transfection reagent. Cells were incubated with the transfection mixture for six hours at 37°C. Following incubation, the transfection media was removed, cells were washed with PBS, and 3 ml of DMEM media supplemented with 10% FBS was added and the cells were allowed to grow overnight in a 37°C, 5% CO_2 incubator.

Sample preparation

After eighteen hours, the media was collected and the cells were washed with 10 ml PBS and lysed in one milliliter of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). A 50 µl sample of lysate and a 150 µl sample of media were removed and resuspended in 50 µl Laemmli sample buffer containing 5% β-mercaptoethanol, heated for ten minutes at 100°C, and separated on a 5% stacking, 10% resolving SDS-polyacrylamide gel.

Immunoblot analysis of sPST and sPST140 proteins

Following SDS-PAGE, proteins were transferred to nitrocellulose membranes at 450 mA overnight. Membranes were then blocked for one hour at 4°C in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). To detect Myc-tagged sPST or sPST140, nitrocellulose membranes were incubated with anti-Myc epitope tag antibody diluted 1:5000 in blocking buffer for two hours at 4°C, and for one hour at 4°C with HRP-conjugated goat anti-mouse IgG secondary antibody diluted 1:4000 in blocking buffer. Membranes were washed twice before and four times after secondary antibody incubation with Tris-buffered saline, pH 8.0, 0.1% Tween 20 for fifteen minutes per wash. Immunoblots were developed using the SuperSignal West Pico chemiluminescence kit and BioExpress Blue Ultra Autorad film.

Competition studies in SW2 cells to evaluate the ability of endogenous PST to polysialylate endogenous NCAM in the presence of a catalytically inactive PST protein competitor

Transfection of SW2 cells on coverslips

SW2 small cell lung carcinoma cells maintained in DMEM, 10% FBS were plated on poly-L-lysine-coated coverslips and grown in a 37°C, 5% CO_2 incubator until 50-70% confluent. Cells on each coverslip were then transfected with one microgram of Myc-tagged PST, sPST or a catalytically inactive full-length (PST H331K) or truncated PST-Myc mutant cDNA in 300 µl OPTI-MEM I containing 3 µl Lipofectamine 2000. Cells were incubated with the transfection mixture for six hours at 37°C followed by the addition of 1 ml DMEM, 10% FBS.

Immunofluorescence staining of SW2 cells

Eighteen hours post-transfection, cells were washed with PBS and permeabilized using -20°C methanol as before. After permeabilization, cells were blocked with immunofluorescence blocking buffer at room temperature for one hour, followed by incubation at room temperature for two hours with anti-Myc epitope tag antibody (1:250 dilution in blocking buffer), to detect expression and localization of PST or a mutant PST, and OL.28 anti-polySia antibody (1:100 dilution) to detect polysialylation. After washing the cells twice with PBS, the cells were then incubated with FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-mouse IgM secondary antibodies in the same manner as detailed above. DAPI staining was not utilized. After washing the cells four times with PBS, the coverslips were mounted on glass microscope slides using 20 µl mounting medium. The amount of polySia on the SW2 cell surface, representative of the level of endogenous NCAM polysialylation, was analyzed using a Zeiss Axiovert 200 M inverted confocal microscope using a 63X oil immersion objective.

Determining the ability of PST and full-length or truncated PST mutants to bind to NCAM

Co-transfection of COS-1 and Lec2 CHO cells on plates

COS-1 and Lec2 CHO cells maintained in DMEM and F-12 media, respectively, supplemented with 10% FBS, were plated onto 100-mm tissue culture plates and grown in a 37°C, 5% CO_2 incubator until 50-70% confluent.

Cells were co-transfected with 10 µg V5-tagged NCAM cDNA and either 10 µg of empty pcDNA3.1/Myc-HisB vector (control), Myc-tagged PST or Myc-tagged PST mutant cDNA in 3 mL OPTI-MEM I containing 30 µl of either Lipofectin (COS-1) or Lipofectamine (Lec2 CHO) transfection reagent. Cells were incubated with the transfection mixture for six hours at 37°C. Following incubation, 7 ml of DMEM (COS-1) or F-12 (Lec2 CHO) media supplemented with 10% FBS was added and the cells were allowed to grow overnight in a 37°C, 5% CO_2 incubator.

Co-immunoprecipitation of NCAM with PST or PST mutants

The following day, cell media was removed and the cells were washed with 10 ml PBS. One milliliter of co-immunoprecipitation buffer (50 mM Hepes, 100 mM NaCl, 1% Triton X-100, pH 7.2) was then added and the cells were scraped off the plates using a sterile cell scraper. The cells were incubated on ice for 20 minutes to allow for lysis, and then pelleted by centrifugation. To immunoprecipitate PST-Myc or a Myc-tagged PST mutant, the resulting supernatant was incubated with 2.5 µl of rabbit polyclonal anti-Myc tag antibody at 4°C for two hours with rotation. Following incubation, 50 µl protein A-Sepharose beads (50% suspension in PBS) was added to each sample and the samples were rotated for one hour at 4°C. The immune complexes bound to protein A-Sepharose beads were pelleted by centrifugation and washed four times with one milliliter of cold co-immunoprecipitation buffer. After washing, the samples were resuspended in 100 µl Laemmli sample buffer containing 5% β-mercaptoethanol and heated to 100°C for ten minutes. Precipitated proteins were separated on a 5% stacking, 10% resolving SDS-polyacrylamide gel and then subjected to immunoblotting as described below.

To determine the relative expression of NCAM and PST or PST mutant proteins in the initial samples, a 150 µl sample of lysate was taken from the supernatant of each centrifuged lysate prior to co-immunoprecipitation. Fifty microliters of Laemmli sample buffer containing 5% β-mercaptoethanol was added to each sample and the samples were heated at 100°C for ten minutes. Prior to immunoblotting, the samples for determining NCAM expression levels were separated on a 5% stacking, 10% resolving SDS-polyacrylamide gel, while those for determining the PST or PST mutant expression levels were separated on a 5% stacking, 15% resolving SDS-polyacrylamide gel.

Immunoblot analysis of NCAM co-immunoprecipitated with PST or PST mutants

Following SDS-PAGE, proteins were transferred to nitrocellulose membranes at 450 mA overnight. Membranes were then blocked for one hour at 4ºC in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). To detect NCAM co-immunoprecipitated with a Myc-tagged PST or PST mutant and determine relative NCAM expression levels, nitrocellulose membranes were incubated with anti-V5 epitope tag antibody diluted 1:5000 in blocking buffer for two hours at 4ºC, and for one hour at 4ºC with HRP-conjugated goat anti-mouse IgG secondary antibody diluted 1:4000 in blocking buffer. To detect relative PST and PST mutant expression levels, membranes were incubated with anti-Myc epitope tag antibody diluted 1:5000 in blocking buffer for two hours at 4ºC, and for one hour at 4ºC with HRP-conjugated goat anti-mouse IgG secondary antibody diluted 1:4000 in blocking buffer. Membranes were washed twice before and four times after secondary antibody incubation with Tris-buffered saline, pH 8.0, 0.1% Tween 20 for 15 minutes per wash. Immunoblots were developed using the SuperSignal West Pico chemiluminescence kit and BioExpress Blue Ultra Autorad film.

Analysis of NCAM, neuropilin-2 and SynCAM 1 polysialylation by wild-type and PBR mutant polyST proteins

Co-transfection of COS-1 cells on plates

COS-1 cells were maintained and transfected on 100-mm tissue culture plates as described earlier. Briefly, COS-1 cells were co-transfected with 10 µg of substrate cDNA (NCAM, neuropilin-2 or SynCAM 1), and 10 µg of either Myc-tagged PST, STX, or a PST or STX PBR mutant. As a control, cells were also transfected with 10 µg of V5-tagged NCAM, neuropilin-2 or SynCAM 1 cDNA, and 10 µg of empty pcDNA3.1/Myc-HisB vector.

Immunoprecipitation of NCAM, neuropilin-2 and SynCAM 1

Eighteen hours post-transfection, cells were washed with 10 ml PBS and lysed in one milliliter of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). The lysates were then pre-cleared with 50 µl protein A-Sepharose beads for one hour at 4ºC with rotation. Following pre-clear, the lysates were briefly centrifuged to remove the beads and then rotated at 4ºC for two hours

with 3 µl anti-V5 epitope tag antibody, followed by incubation with 50 µl protein A-Sepharose beads for one hour at 4ºC. After incubation, the beads were washed four times with immunoprecipitation buffer and once with immunoprecipitation buffer containing 1% SDS. Samples were then resuspended in 50 µl Laemmli sample buffer containing 5% β-mercaptoethanol, heated for ten minutes at 65ºC, and separated on a 3% stacking, 5% resolving SDS-polyacrylamide gel. The relative expression levels of NCAM, neuropilin-2, SynCAM 1, and PST/STX proteins were evaluated as described above.

Immunoblot analysis of NCAM, neuropilin-2, and SynCAM 1 polysialylation

The polysialylation of NCAM, neuropilin-2 and SynCAM 1 by wild-type and mutant PST and STX proteins was evaluated with OL.28 immunoblotting. Briefly, proteins were transferred to a nitrocellulose membrane at 450 mA overnight. Membranes were blocked for one hour at 4ºC in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). Membranes were then incubated overnight at 4ºC with OL.28 anti-polySia antibody diluted 1:150 in 2% nonfat dry milk in Tris-buffered saline, pH 8.0, followed by a one hour incubation at 4ºC with HRP-conjugated goat anti-mouse IgM antibody diluted 1:4000 in blocking buffer. Membranes were then washed and developed as described above.

Triple transfection of COS-1 cells with a polySia substrate, wild-type polyST, and mutant polyST

COS-1 cells were prepared and transfected as described above. Briefly, COS-1 cells on 100-mm tissue culture plates were transfected with 2.5 µg of V5-tagged polySia substrate cDNA (NCAM, neuropilin-2 or SynCAM 1), 2.5 µg PST- or STX-Myc cDNA, and 15 µg of a Myc-tagged PST or STX mutant cDNA. As a control, cells were also transfected with two micrograms each of V5-tagged polySia substrate and PST- or STX-Myc cDNA, and 15 µg of empty pcDNA3.1/Myc-HisB vector.

Evaluation of a catalytically inactive polyST PBR mutant's ability to compete with wild-type polyST and reduce substrate polysialylation

In this *in vitro* competition assay, V5-tagged NCAM, neuropilin-2 or SynCAM 1 is expressed in COS-1 cells along with PST- or STX-Myc, and a catalytically inactive full-length PST or STX protein in a 1:1:6 ratio as

described immediately above for a triple transfection. The template cDNA for all of the created catalytically inactive polyST PBR mutants are PST H331K and STX H346K, which contain a single point mutation in the very small sialyl motif that renders the protein unable to synthesize polySia.

Immunoprecipitation of NCAM, neuropilin-2 and SynCAM 1

Eighteen hours post-transfection, cells were washed with 10 ml PBS and lysed in one milliliter of immunoprecipitation buffer. The lysates were pre-cleared with 50 µl protein A-Sepharose beads for one hour at 4°C with rotation, then incubated with 3 µl anti-V5 tag antibody followed by 50 µl protein A-Sepharose beads as described above. Beads were washed four times with immunoprecipitation buffer and once with immunoprecipitation buffer containing 1% SDS. Samples were then resuspended in 50 µl Laemmli sample buffer containing 5% β-mercaptoethanol, heated for ten minutes at 65°C, and separated on a 4-15% Mini-PROTEAN TGX precast gel. The relative expression levels of NCAM, neuropilin-2, SynCAM 1, and PST/STX wild-type and mutant proteins were evaluated as described above, utilizing the 4-15% Mini-PROTEAN TGX precast gel for separating the proteins.

Evaluation of polyST competition through analysis of substrate polysialylation

Proteins were transferred to a nitrocellulose membrane at 400 mA overnight. Membranes were blocked for one hour at 4°C in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). Membranes were then incubated overnight at 4°C with 12F8 anti-polySia antibody diluted 1:100 in 2% nonfat dry milk in Tris-buffered saline, pH 8.0, followed by a one hour incubation at 4°C with HRP-conjugated goat anti-rat IgM antibody diluted 1:2500 in blocking buffer. Membranes were then washed and developed as described above.

Evaluation of the ability of PST H331K, STX H346K, and ST8Sia III to compete with wild-type PST and reduce NCAM polysialylation

V5-tagged NCAM was expressed in COS-1 cells along with PST-Myc, and a catalytically inactive full-length PST or STX protein, or ST8Sia III in a 1:1:6 ratio as described previously. Eighteen hours post-transfection, cells were washed with 10 ml PBS and lysed in one milliliter of immunoprecipitation buffer. The lysates were pre-

cleared with 50 µl protein A-Sepharose beads for one hour at 4ºC with rotation, then incubated with 3 µl anti-V5 tag antibody followed by 50 µl protein A-Sepharose beads as described above. Beads were washed, samples were resuspended in 50 µl Laemmli sample buffer, heated for ten minutes at 65ºC, and separated on a 4-15% Mini-PROTEAN TGX precast gel as before. The relative expression levels of NCAM and PST wild-type and mutant proteins or ST8Sia III were evaluated as described above. Proteins were then transferred to a nitrocellulose membrane and immunoblotting with V5- and Myc-epitope tag antibody, as well as OL.28 anti-polySia antibody, was performed as described previously.

Determining the ability of STX and STX H346K to bind to NCAM

COS-1 and cells maintained in DMEM, supplemented with 10% FBS, were plated onto 100-mm tissue culture plates and grown in a 37ºC, 5% CO_2 incubator until 50-70% confluent. Cells were co-transfected with 10 µg V5-tagged NCAM cDNA and either 10 µg of empty pcDNA3.1/Myc-HisB vector (control), or Myc-tagged PST, PST H331K, STX, or STX H346K cDNA in 3 mL OPTI-MEM I containing 30 µl Lipofectin transfection reagent. Cells were incubated with the transfection mixture for six hours at 37ºC. Following incubation, 7 ml of DMEM media supplemented with 10% FBS was added and the cells were allowed to grow overnight in a 37ºC, 5% CO_2 incubator. Co-immunoprecipitation and immunoblotting to detect NCAM bound to PST or STX proteins was performed identically as above under “Determining the ability of PST and full-length or truncated PST mutants to bind to NCAM”.

CHAPTER III

IDENTIFICATION OF A SPECIFIC REGION IN ST8SIA IV/PST THAT IS REQUIRED FOR THE RECOGNITION OF NEURAL CELL ADHESION MOLECULE

Parts of this chapter were originally published in The Journal of Biological Chemistry. Zapater, J.L., and Colley, K.J. Sequences Prior to Conserved Catalytic Motifs of Polysialyltransferase ST8Sia IV Are Required for Substrate Recognition. J. Biol. Chem. 2012; 287:6441-53. © the American Society for Biochemistry and Molecular Biology.

Introduction

Current evidence suggests that the interaction of NCAM with the polySTs involves an initial recognition step that is mediated by protein-protein interactions. Using a soluble form of PST that consisted of residues 40-359, Angata *et al.* [321] constructed a series of chimeras in which regions of the soluble PST molecule were substituted with the corresponding regions of ST8Sia III. ST8Sia III is a related α2, 8-sialyltransferase that is capable of autopolysialylation but does not effectively polysialylate NCAM [322]. When PST residues 40-82, which are prior to the catalytic sialylmotifs, were replaced with the corresponding region of ST8Sia III, NCAM polysialylation was significantly decreased by approximately sixty percent, despite the fact that enzyme autopolysialylation remained comparable to wild-type PST. This result suggested that there was a defect in the chimera's ability to recognize the NCAM substrate, rather than a defect in overall enzyme catalytic function.

In a further study, Angata *et al.* [321] created a chimera in which residues 128-359 (to the C-terminus) of PST were exchanged for the corresponding region in ST8Sia III and incubated it *in vitro* with wild-type soluble PST, soluble NCAM, and CMP-sialic acid. In the presence of increasing concentrations of the chimera, NCAM polysialylation significantly decreased, suggesting that the chimera, containing amino acids 40-128 of PST, was competing with wild-type PST for recognition of NCAM, hence preventing the polysialylation of NCAM by full length, catalytically active PST. Since wild-type ST8Sia III did not reduce NCAM polysialylation by PST, it is possible that PST sequences 40-128 are responsible for the competition.

Recent work from our laboratory supports the above finding [305]. We found that replacement of specific residues in the PBR of PST (amino acids 71-105) with alanines, namely Arg^{82} and Arg^{93}, significantly reduced or eliminated NCAM polysialylation when mutated singly or doubly, respectively. Meanwhile, examination of PST

autopolysialylation indicated that this process was not affected, indicating a loss of the ability of PST to specifically polysialylate NCAM rather than a loss of overall catalytic activity of the enzyme.

Cumulatively, these studies above indicated to us that there is a region prior to the large sialylmotif in PST that is necessary for the effective polysialylation of NCAM. This is an interesting finding because these residues do not fall within the PST catalytic region, which begins with the start of the large sialylmotif at residue 141 and which is responsible for polySia synthesis. As mentioned previously, our hypothesis is that substrate polysialylation is a highly protein-specific process, requiring an initial polyST-substrate interaction that is mediated by protein-protein interactions. Combined with the above work, I further hypothesized that the membrane proximal region prior to the large sialylmotif in PST, which consists of the first 140 residues, contains amino acid sequences that may mediate this initial protein-protein interaction. I therefore focused on PST residues 1-140 and examined this portion of PST for a region that specifically functions for NCAM recognition.

Experimental Approach

To determine if residues 1-140 of PST contain sequences required for NCAM recognition, I decided to use an *in vivo* cellular competition approach that is summarized in Figure 9. The premise of this competition approach is that a catalytically-inactive full-length or truncated PST protein, which still contains sequences that are required for NCAM recognition, will compete with wild-type catalytically-active PST by binding to the NCAM recognition sequences and thus reduce or block NCAM polysialylation by the wild type enzyme. Should a PST mutant not contain sequences that are required for NCAM recognition, then I would not expect a reduction in NCAM polysialylation. For my competition studies, I utilized SW2 small cell lung carcinoma cells. These cells provide the appropriate cellular environment for studying NCAM recognition and subsequent polysialylation because they contain endogenous NCAM, endogenous wild-type PST, and the machinery necessary for polySia synthesis (CMP-sialic acid and the CMP-sialic acid transporter). Furthermore, SW2 cells exhibit cell surface polySia staining with OL.28 anti-polySia antibody, indicating polysialylation of the NCAM in the basal state (Figure 10).

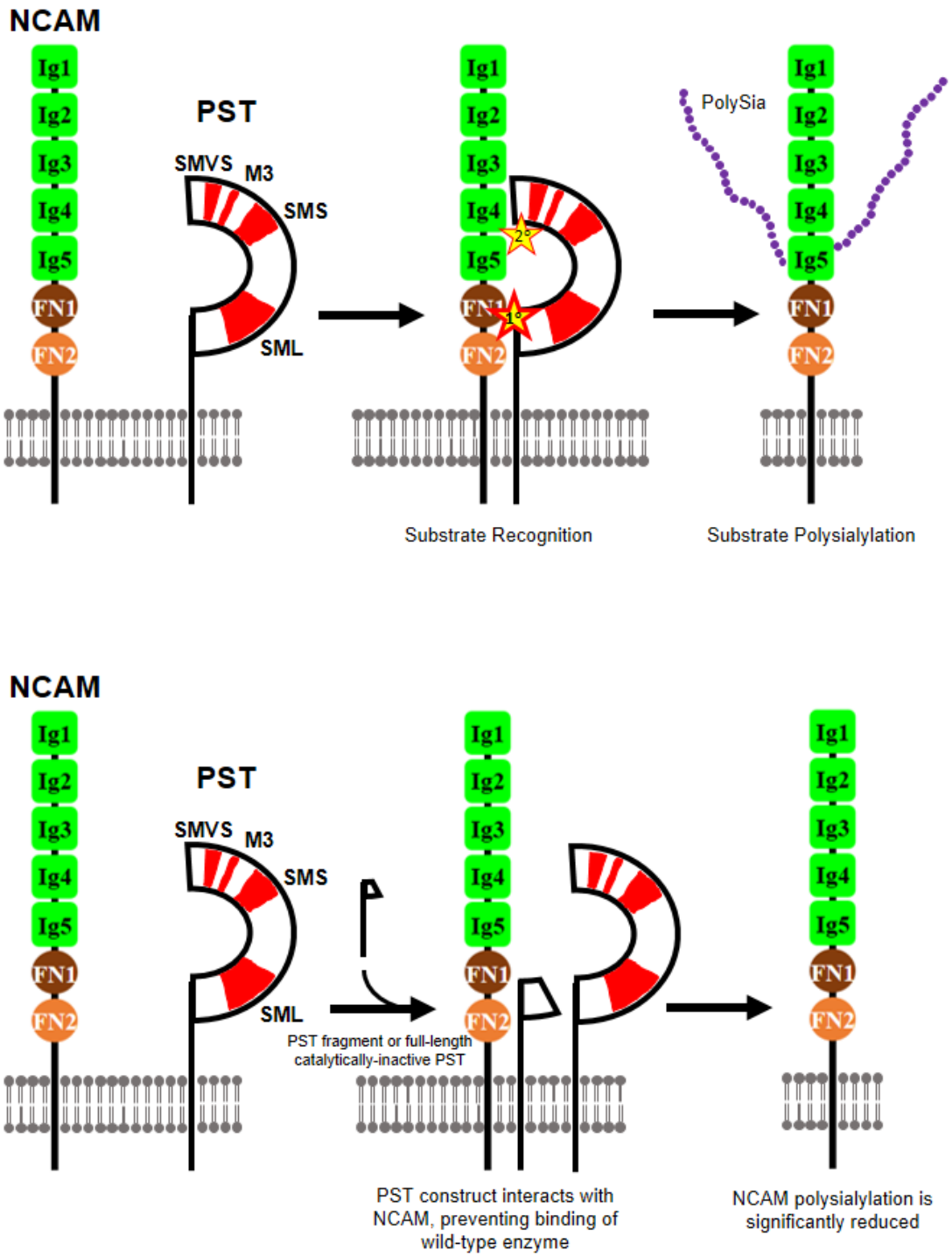

Figure 9. Schematic overview of polyST competition assay. We hypothesize that initial polyST-substrate protein-protein interactions are required to stabilize the polyST next its substrate and promote effective polysialylation (*top*). In our competition assay, we predict that introduction in excess of a catalytically-inactive PST mutant protein that still contains sequences required for NCAM recognition will interfere with NCAM polysialylation by preventing the interaction between NCAM and wild-type PST (*bottom*).

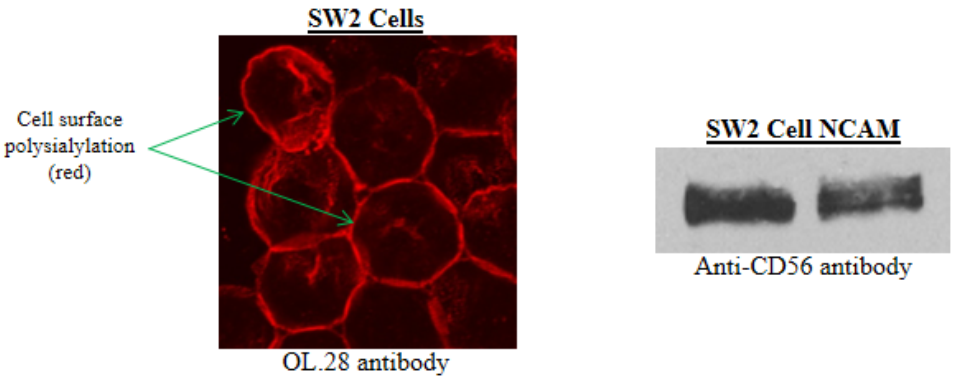

Figure 10. Presence of cell surface polysialylation and NCAM in small cell lung carcinoma cells. To examine the presence of polysialylation in SW2 cells (*left panel*), these cells first were plated on coverslips and incubated in a 5% CO_2, 37°C incubator for 18 hours. After incubation, cells were washed with PBS, fixed with cold methanol, then incubated for two hours with OL.28 anti-polySia antibody, followed by incubation for one hour with TRITC-conjugated goat anti-mouse IgM secondary antibody. Coverslips were mounted on microscope slides, then viewed using a Zeiss 200M inverted confocal microscope, 40X objective. OL.28 anti-polySia staining, indicative of polysialylation, is seen on the SW2 cell surface (*red*) indicating the presence of polysialylated NCAM. To assay for the presence of NCAM (*right panel*), SW2 cells were grown on 100-mm tissue culture plates in a 5% CO_2, 37°C incubator for 18 hours. After incubation, cells were lysed, and a sample of lysate was removed, electrophoresed on an SDS-PAGE gel, followed by immunoblotting with anti-CD56 primary antibody for 2 hours, followed by goat anti-mouse IgG secondary antibody for one hour. The two lanes shown above show the experiment performed in duplicate.

A series of PST mutants were created for competition studies (Figure 11), as well as co-immunoprecipitation studies. One catalytically-active PST protein that did not contain the cytosolic tail or transmembrane region (sPST) was created to determine the effect of these regions on NCAM recognition and polysialylation. A full-length catalytically-inactive PST protein (PST H331K) was created by mutating His^{331} to lysine as was done previously [302]. PST H331K serves as the baseline for competition studies since it is catalytically inactive and contains all PST residues, thus competition from this PST mutant would indicate that PST does contain sequences critical for NCAM recognition. Later I will use the PST H331K mutant as a template to identify specific residues involved in substrate recognition (Chapters IV and V). To closely investigate the region prior to the large sialylmotif, I created a PST protein consisting of only the first 140 residues of PST (PST140) as well as its soluble counterpart (sPST140). These two PST mutants do not contain any catalytic regions and hence have no polysialylation activity. I then created PST mutants that were truncated backward from residue 140 based on evidence from Angata *et al.* [321] or Foley *et al.* [305], and include PST mutants consisting of the first 127, 105, 71, and 62 residues of PST (PST127, PST105, PST71, and PST62). PST127 and PST62 are based on previous chimera analyses [321], whereas PST105 and PST71 represent the C-terminal and N-terminal ends of the PBR [305], respectively.

For competition studies, each PST mutant was transiently expressed in SW2 cells individually, and the resultant change in endogenous NCAM cell surface polysialylation was evaluated to determine the ability of each PST protein to recognize NCAM and compete with endogenous catalytically active PST. Furthermore, co-immunoprecipitation analyses were also utilized to specifically determine enzyme-substrate binding. Here, PST or a PST mutant was expressed individually in COS-1 cells with NCAM. Following lysate preparation, each enzyme was immunoprecipitated and NCAM binding was examined with immunoblotting.

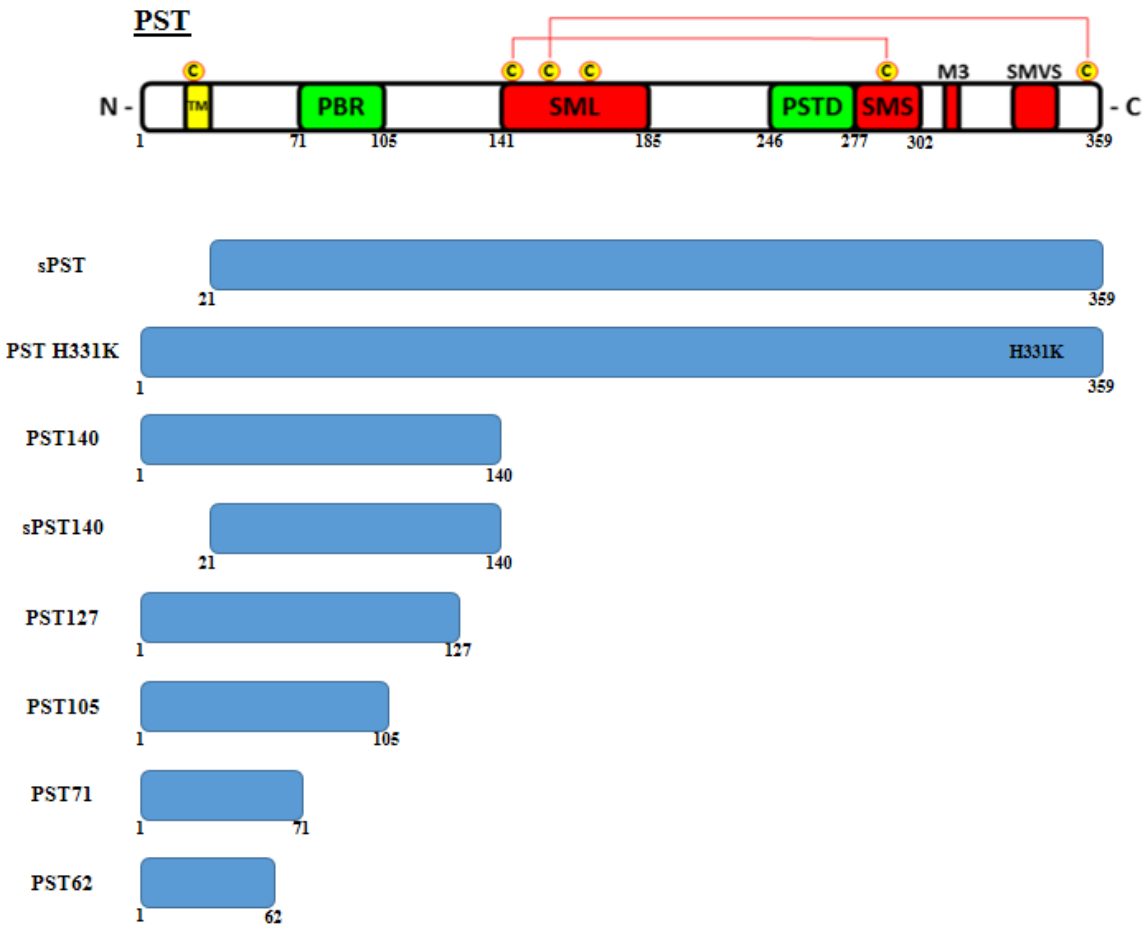

Figure 11. Schematic of soluble PST and catalytically-inactive full-length and truncated PST mutants. The creation of these PST constructs is detailed in Chapter II. A soluble PST protein consisting of the entire Golgi lumenal region of PST was initially created to determine the effects of the cytosolic tail and transmembrane region on NCAM recognition and polysialylation. A full-length catalytically-inactive PST protein was created by mutating His^{331} to lysine as done previously [302], and serves as a template protein for competition. All catalytically-inactive, truncated PST proteins were created based on previous data [305, 321] to narrow down a region prior to the large sialylmotif that is critical for NCAM recognition.

Results

All catalytically-inactive PST mutants express and localize to the Golgi apparatus except for PST105

The polySTs are glycosyltransferases that localize to the Golgi apparatus, which is the site of polySia addition onto target glycoproteins. Therefore, it is critical that all created PST mutants also similarly localize to the Golgi apparatus, as this would indicate proper folding and trafficking through the secretory pathway. This is especially important here as I made very drastic protein truncations. Myc-tagged wild-type PST, soluble PST (sPST), and each PST mutant were expressed individually in COS-1 cells, which were then stained with anti-Myc antibody to examine the cellular localization of these proteins (Figure 12, *Localization*). To examine enzyme autopolysialylation, which should only occur in catalytically active enzymes, the COS-1 cells were also stained with OL.28 anti-polySia antibody (Figure 12, *Autopolysialylation*).

I found that similar to wild-type PST, the membrane-associated PST mutants were found to be localized primarily to the Golgi apparatus with the exception of PST105, which did not demonstrate any expression. It is possible that this truncated PST may become unstable and is thus quickly degraded. Therefore, PST105 was not utilized further in any competition or co-immunoprecipitation studies. Similar to the membrane-bound PST mutants other than PST105, sPST and sPST140 are found in the Golgi apparatus as they transit through the secretory pathway. Furthermore, when expressed in COS-1 cells, PST and sPST were autopolysialylated as is expected for active polySTs, whereas PST H331K and all truncated PST mutant proteins, which lack critical catalytic sequences, are not.

Soluble PST (sPST) and PST140 (sPST140) are secreted from COS-1 cells

As shown above, sPST and sPST140 are localized primarily to the Golgi apparatus, but since they are soluble proteins, their appearance in the Golgi should be a result of their temporary presence here, as these two PST mutants should transit through the entire secretory pathway. I therefore sought to determine if these two PST mutants are also successfully secreted from COS-1 cells. For this, I expressed sPST and sPST140 in COS-1 cells and collected a sample of lysate (protein expression) and media (protein secretion) for expression analysis using

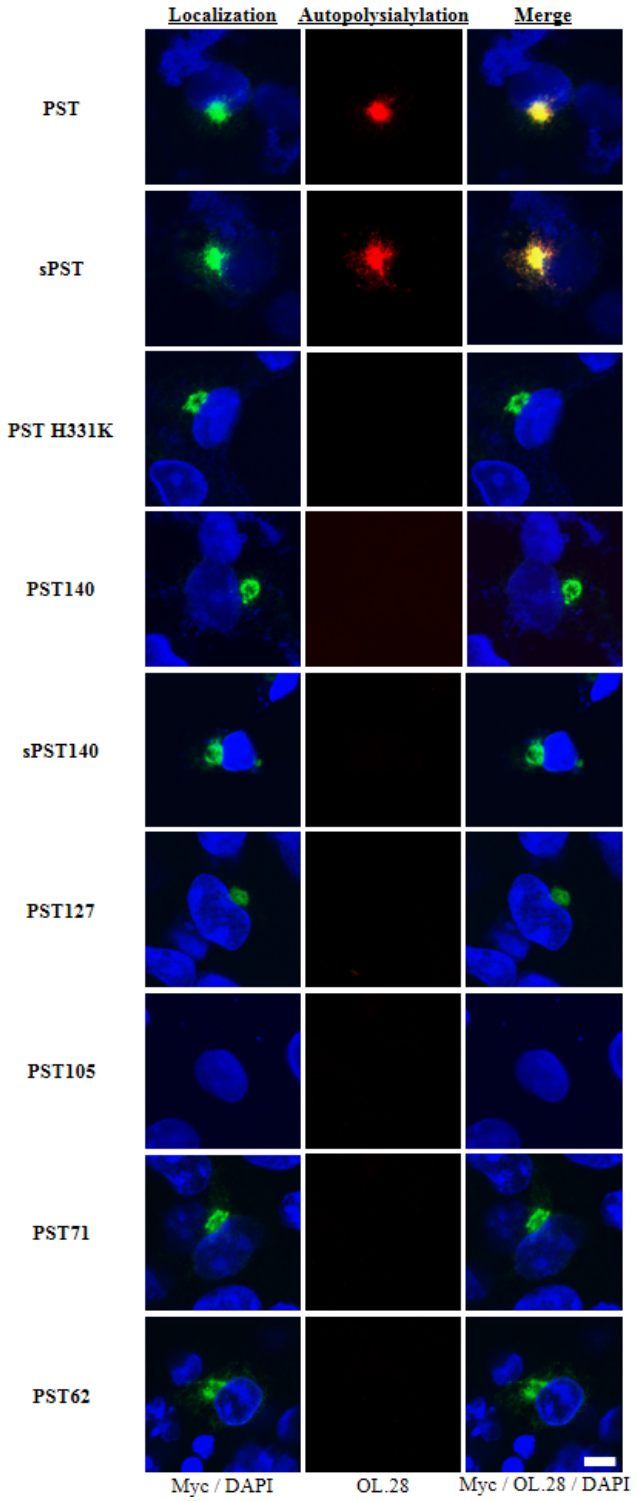

Figure 12. All PST mutants are predominantly localized to the Golgi apparatus except PST105. Myc-tagged PST, sPST, or a PST mutant were expressed in COS-1 cells as described before. Cells were then washed, fixed with methanol, and stained with anti-Myc antibody (*Localization, green*) to determine protein localization, and OL.28 anti-polySia antibody (*Autopolysialylation, red*) to detect autopolysialylation (enzyme activity). DAPI staining indicates the location of the nucleus (*Localization and Merge, blue*). Scale bar = 10 μm.

These data were previously published in: Zapater, J.L., and Colley K.J. (2012) Sequences Prior to Conserved Catalytic Motifs of Polysialyltransferase ST8Sia IV Are Required for Substrate Recognition. *J. Biol. Chem.* 287:6441-53.

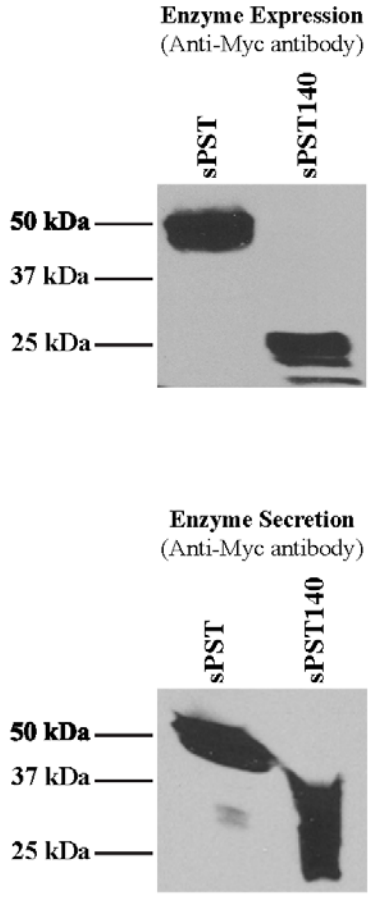

Figure 13. Soluble PST (sPST) and PST140 (sPST140) are secreted from COS-1 cells. Myc-tagged sPST or sPST140 were expressed in COS-1 cells as described in Chapter II. Eighteen hours post-transfection, the cell media was collected and the cells were lysed. A 50 µl sample of lysate and a 150 µl sample of media were then subjected to SDS-PAGE and immunoblotting with anti-Myc epitope antibody to examine protein expression (*top panel*) and secretion (*bottom panel*). Note that the sample size from the media is three times greater than that from lysate. This is because cell lysates were created in one milliliter of buffer, whereas a total of three milliliters of cell media was collected.

anti-Myc epitope tag antibody (Figure 13). We found that in addition to their expression in COS-1 cells (Figure 13, *top panel*), sPST and sPST140 are indeed secreted from these cells (Figure 13, *bottom panel*). Since these soluble proteins express similarly to wild-type PST (Figure 12) and are secreted from cells (Figure 13), sPST and sSPT140 were utilized in competition studies.

Expression of PST H331K, PST140 and sPST140 blocks NCAM polysialylation by endogenous PST in SW2 cells

To determine whether PST H331K, PST140, and sPST140 could compete with endogenous PST and decrease NCAM polysialylation, each protein was individually expressed in SW2 cells (Figure 14). The cells were then stained with anti-Myc antibody to identify cells expressing the Myc-tagged PST proteins (Figure 14, *Localization*), and OL.28 anti-polySia antibody to examine the polysialylation of endogenous NCAM (Figure 14, *Polysialylation*). We found that SW2 cells expressing exogenous PST or sPST displayed an increase in cell surface NCAM polysialylation and Golgi enzyme autopolysialylation relative to surrounding cells that did not express these exogenous proteins (Figure 14, *PST* and *sPST*, arrowheads). We were only able to detect enzyme autopolysialylation when exogenous PST or sPST are overexpressed, which likely suggests that SW2 cells express low levels of endogenous PST.

In contrast, SW2 cells expressing PST H331K demonstrate substantially reduced NCAM polysialylation compared with nonexpressing cells (Figure 14, *PST H331K*, arrowheads). Likewise, SW2 cells expressing PST140 (Figure 14, *PST140*, arrowheads), although fewer in number, exhibited a similar decrease in surface NCAM polysialylation. Lastly, the expression of sPST140 in SW2 cells also reduced surface NCAM polysialylation, although residual OL.28 staining was observed in some expressing cells (Figure 14, *sPST140*, arrowheads). This may be due to the transient residence of sPST140 in the Golgi apparatus, as this is a soluble protein. These results support the notion that PST residues 21-140 between the end of the transmembrane region and the beginning of the large sialylmotif are likely to function in NCAM recognition.

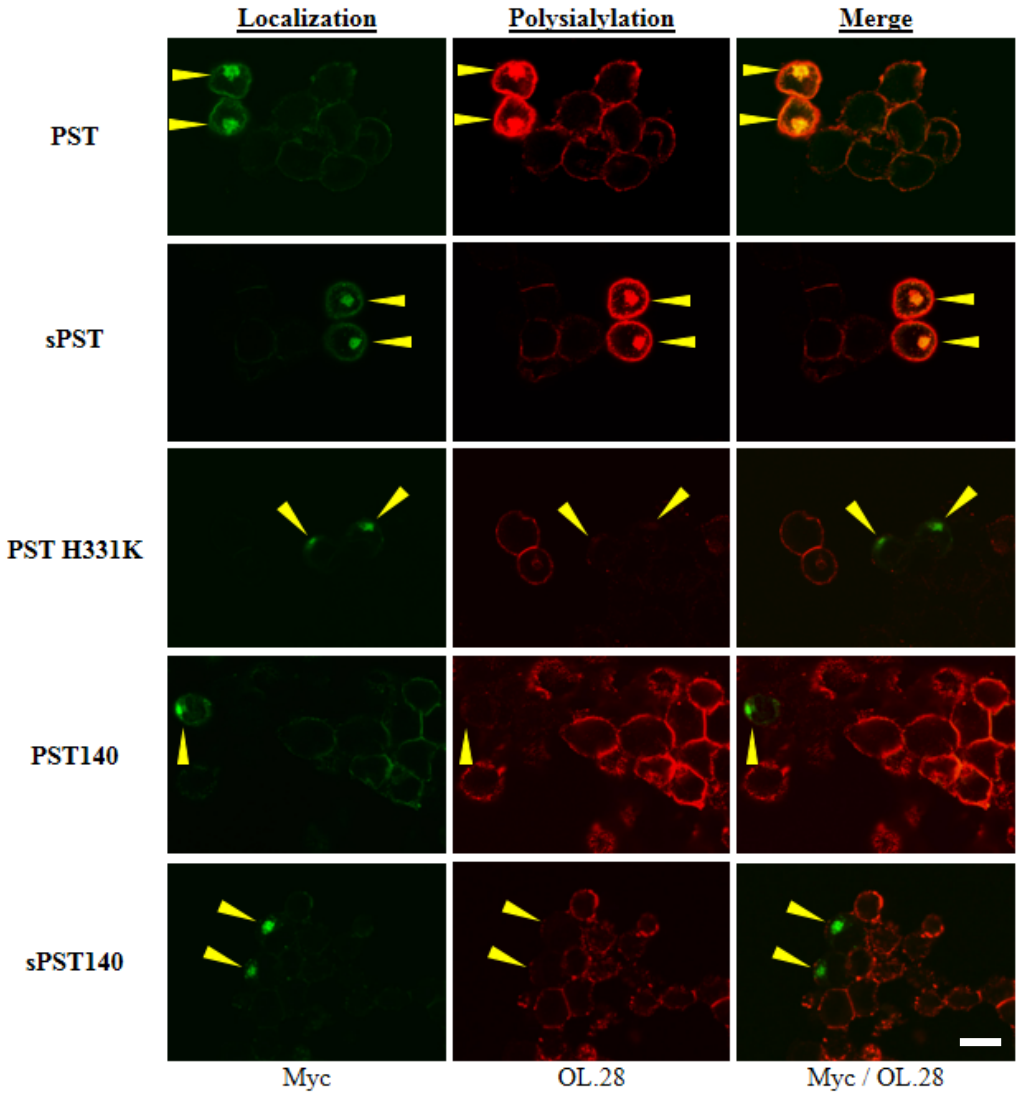

Figure 14. Expression of PST H331K, PST140, and sPST140 inhibits NCAM polysialylation by endogenous PST in SW2 cells. Myc-tagged PST, sPST, PST H331K, PST140, and sPST140 were transiently expressed in SW2 cells containing endogenous PST and polysialylated NCAM. Eighteen hours post-transfection, cells were fixed and indirect immunofluorescence was performed using anti-Myc antibody to analyze exogenous enzyme localization (*Localization*), and OL.28 anti-polySia antibody to analyze total polysialylation (*Polysialylation*). Overlaying the fluorescent signals for PST protein localization and polysialylation demonstrated the impact that expressing exogenous PST proteins had on the polysialylation of endogenous SW2 cell NCAM (*Merge*). Cells expressing exogenous PST mutant proteins are marked with yellow arrowheads. Cells were examined using a Zeiss 200 M inverted confocal microscope, 63X oil immersion objective. Scale bar = 10 μm.

These data were previously published in: Zapater, J.L., and Colley K.J. (2012) Sequences Prior to Conserved Catalytic Motifs of Polysialyltransferase ST8Sia IV Are Required for Substrate Recognition. *J. Biol. Chem.* 287:6441-53.

Expression of PST127, but not PST71 or PST62, blocks NCAM polysialylation by endogenous PST in SW2 cells, suggesting that residues 71-127 in PST are critical for NCAM recognition

To narrow down the region involved in NCAM recognition, I utilized our PST mutants that were truncated backward from residue 140 of PST, namely, PST127, PST71 and PST62. As mentioned earlier, PST105 was not utilized due to a lack of protein expression. Of the remaining three truncated proteins, PST127 contains the PBR, which extends from residues 71 to 105, whereas PST71 and PST62 do not. Each of these PST mutants was expressed individually in SW2 cells and their impact on endogenous NCAM polysialylation was analyzed as described above (Figure 15).

It should be noted that similar to PST140, PST127, PST71 and PST62 were detected in fewer SW2 cells than full-length PST or PST H331K, suggesting some instability or increased turnover of these drastically truncated proteins. Nevertheless, I found that cells expressing PST127 exhibited a decrease in NCAM polysialylation that was similar to that seen for PST140 (Figure 15, *PST140* and *PST127*, arrowheads). In contrast, cells expressing PST71 or PST62 did not show a decrease in cell surface NCAM polysialylation (Figure 15, *PST71* and *PST62*, arrowheads). Instead, the polysialylation of NCAM seemed to be unchanged from baseline levels, suggesting that PST71 and PST62 do not have sequences required for NCAM recognition and are thus unable to compete with endogenous PST to block NCAM polysialylation. Collectively, my SW2 cell competition studies suggest that residues 71-127 of PST are important for NCAM recognition.

The ability of catalytically-inactive PST mutants to bind to NCAM correlates with their ability to competitively inhibit NCAM polysialylation

Previous work from the laboratory demonstrated that PST binds to NCAM [314]. I therefore wanted to determine whether the ability of PST H331K, PST140, sPST140, and PST127 to significantly reduce or eliminate NCAM polysialylation in SW2 cells is the result of their ability to bind to NCAM and block access of endogenous, catalytically active PST. To evaluate this possibility, I took a co-immunoprecipitation approach. I first co-expressed V5-tagged NCAM with Myc-tagged wild-type PST, sPST, PST H331K, PST140 or sPST140 in COS-1 cells. The

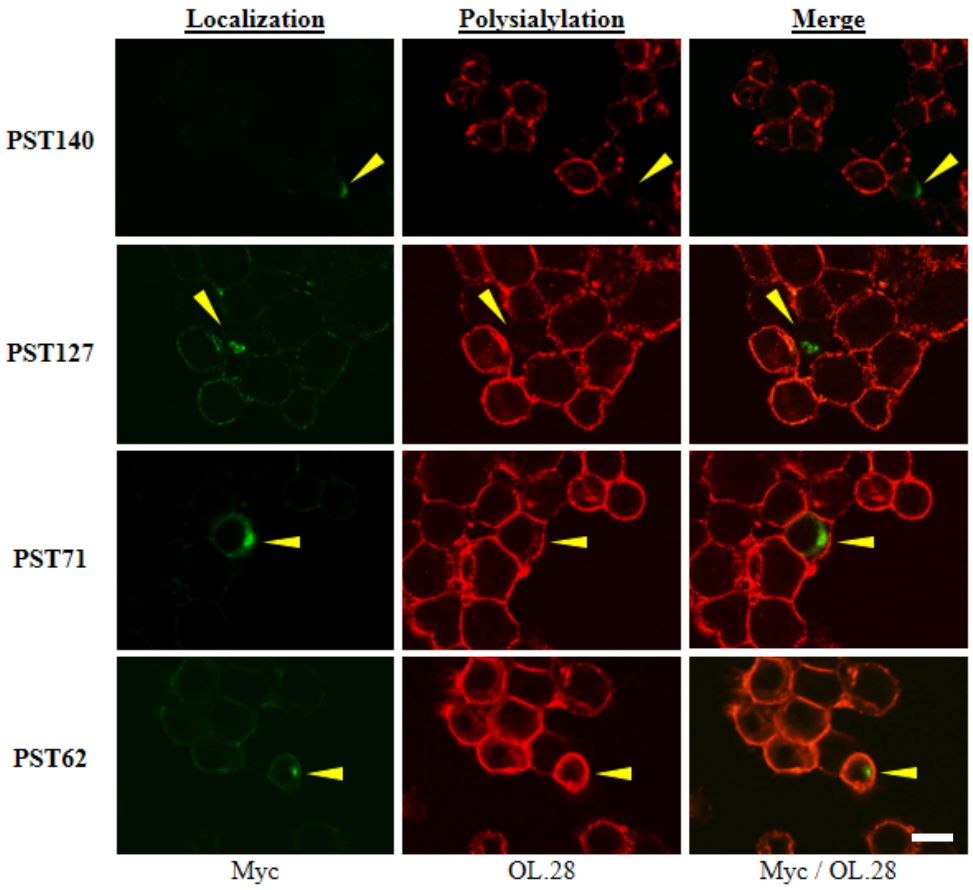

Figure 15. SW2 cell NCAM polysialylation by endogenous PST is inhibited by the expression of PST127 but not PST71 or PST62. Myc-tagged PST140, PST127, PST71 and PST62 were transiently expressed in SW2 cells. Eighteen hours post-transfection, cells were fixed and indirect immunofluorescence was performed to analyze exogenous enzyme protein localization (*Localization*) or total polysialylation (*Polysialylation*). Overlaying the fluorescent signals for PST protein localization and polysialylation demonstrated the impact that expressing exogenous PST proteins had on the polysialylation of endogenous SW2 cell NCAM (*Merge*). Cells expressing exogenous PST proteins are marked with yellow arrowheads. Cells were examined using a Zeiss 200 M inverted confocal microscope, 63X oil immersion objective. Scale bar = 10 μm.

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enzymes were then immunoprecipitated from the cell lysates with an anti-Myc antibody, and co-immunoprecipitated NCAM-V5 was then detected by immunoblotting with anti-V5 antibody (Figure 16, *top panel*). Relative expression levels of NCAM and PST proteins were determined by immunoblotting an aliquot of cell lysate with anti-V5 and anti-Myc antibodies, respectively (Figure 16, *middle and bottom panels*).

I found a small amount of nonspecific binding of NCAM to the protein A-Sepharose beads (see Chapter II) in the absence of co-expressed enzyme (Figure 16, *top panel, lane 1*). As expected however, NCAM-V5 was co-immunoprecipiated with PST (Figure 16, *top panel, lane 2*). Additionally, NCAM-V5 was co-immunoprecipitated with sPST, suggesting that the cytosolic tail and transmembrane region of PST are not critical for NCAM binding (Figure 16, *top panel, lane 5*). I also observed that NCAM-V5 also bound to Myc-tagged, membrane associated PST H331K, PST140, as well as sPST140 (Figure 16, *top panels, lanes 3, 4, and 6*). These results correlate with our competition studies, and indicate that the ability of PST mutant proteins to compete with endogenous PST in SW2 cells is due to their ability to bind to NCAM. Furthermore, these results suggest that residues 21-140 of PST contains a binding site for NCAM interaction.

I next performed a similar co-immunoprecipitation analysis in Lec2 CHO cells that compared the binding of NCAM to PST and all of the truncated PST mutant proteins (Figure 17). Lec2 CHO cells do not have a functional CMP-sialic acid transporter, and hence cannot add sialic acid or polySia to glycoconjugates [326]. I used these Lec2 CHO cells to eliminate the potential effects of polySia on binding of wild-type PST and NCAM. I found that similar to full-length PST, PST140 and PST127 bound to NCAM (Figure 17, *top panels, lanes 2-4*), which is consistent with their ability to block NCAM polysialylation in SW2 cells. In contrast, PST71 and PST62 exhibited a significantly reduced ability to bind to NCAM (Figure 17, *top panels, lanes 5 and 6*), even when their somewhat lower level of expression is taken into consideration (Figure 17, *bottom panels, lanes 5 and 6*). This result correlates with the inability of PST71 and PST62 to block NCAM polysialylation in SW2 cells.

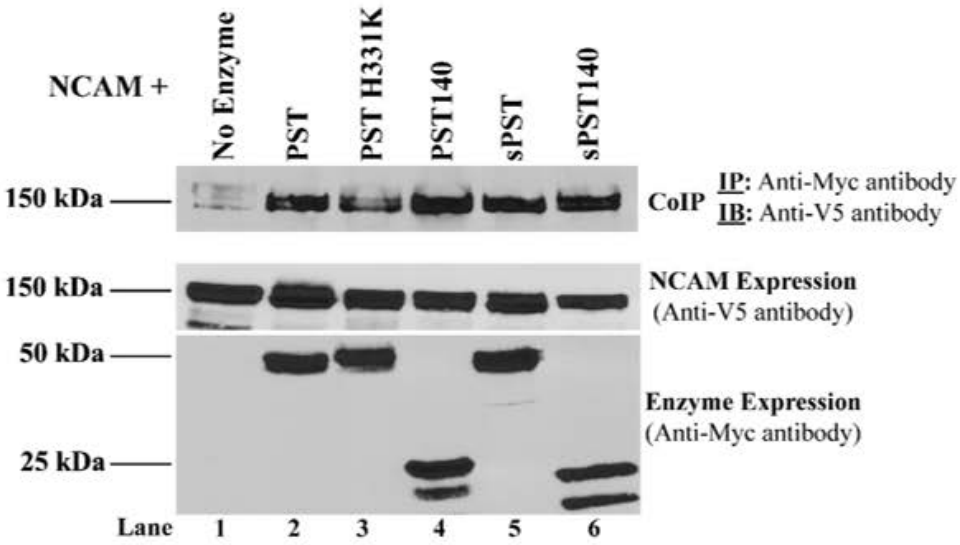

Figure 16. Residues 21-140 of PST contain sequences that are critical for NCAM binding. V5-tagged NCAM was co-expressed with Myc-tagged PST, PST H331K, PST140, sPST, or sPST140 in COS-1 cells. After 18 h, cells were lysed and lysates were incubated with anti-Myc epitope tag antibody to immunoprecipitate the PST proteins, followed by incubation with protein A-Sepharose beads for one hour. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-V5 antibody to detect co-immunoprecipitated NCAM (*top panel*). To determine the relative expression levels of NCAM and PST proteins, an aliquot of lysate was removed prior to co-immunoprecipitation and subjected to SDS-PAGE and immunoblotting with anti-V5 antibody to detect NCAM (*middle panel*) and anti-Myc antibody to detect the PST proteins (*bottom panel*).

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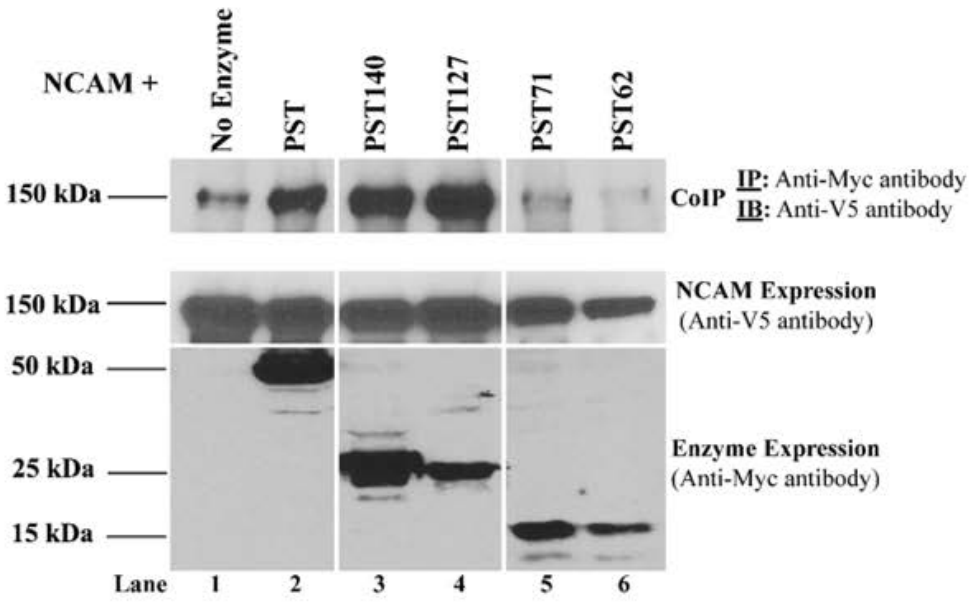

Figure 17. Similarly to wild-type PST, PST140 and PST127 effectively bind to NCAM, whereas PST71 and PST62 do not. V5-tagged NCAM was co-expressed with Myc-tagged PST, PST140, PST127, PST71, or PST62 in Lec2 CHO cells. After 18 h, cells were lysed and lysates were incubated with anti-Myc epitope tag antibody to immunoprecipitate the PST proteins, followed by incubation with protein A-Sepharose beads for one hour. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-V5 antibody to detect co-immunoprecipitated NCAM (*top panel*). To determine the relative expression levels of NCAM and PST proteins, an aliquot of lysate was removed prior to co-immunoprecipitation and subjected to SDS-PAGE and immunoblotting with anti-V5 antibody to detect NCAM (*middle panel*) and anti-Myc antibody to detect the PST proteins (*bottom panel*).

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Discussion

Earlier polysialylation and binding studies focused on the NCAM FN1 domain. Deletion or replacement of this domain, or the mutation of specific residues in an acidic surface patch, indicated that this domain was required for effective polysialylation of N-glycans on the adjacent Ig5 domain [311-314]. From these NCAM studies, it was first postulated that specific sequences within NCAM FN1 served as a specific substrate recognition site for the polySTs. Since there is no polySia addition at the NCAM FN1 domain, this interaction at NCAM FN1 is likely to be a result of a protein-protein interaction and not a protein-glycan interaction. Furthermore, and more importantly, if substrate polysialylation is indeed substrate-specific and utilizes specific sequences within the NCAM FN1 domain, then there should be complementary sequences within the polySTs that would also be involved in this interaction. Through analysis of PST/ST8Sia III chimeric proteins, Angata *et al.* [321] was one of the first groups to provide evidence supporting this theory. They found that the proximal Golgi lumenal region prior to the large sialylmotif, which is not part of the catalytic region of PST, was important for NCAM polysialylation. Previous work from our laboratory seconded this work, as we identified a polybasic region in the PST within this proximal Golgi lumenal region that contained residues that were critical for NCAM polysialylation [305]. Through competition and co-immunoprecipitation studies, the work presented here provides direct evidence that the proximal Golgi lumenal region of PST prior to the large sialylmotif is critical for recognition of the NCAM substrate.

The reduction in polysialylation of SW2 cell surface NCAM seen in the presence of PST H331K, a catalytically-inactive full-length PST protein, suggests that PST contains amino acid sequences that are important for NCAM recognition (Figure 14). Furthermore, the ability of PST140 and sPST140 to similarly reduce SW2 cell NCAM polysialylation suggests that the region between the end of the transmembrane region and the start of the large sialylmotif of PST, encompassing residues 21-140, contains a sequence that is important for NCAM recognition (Figure 14). This finding alone correlates with NCAM polysialylation work from previous studies detailed earlier. Furthermore, I was able to use competition studies to narrow down this region to residues 71-127, as PST127 reduced SW2 cell surface NCAM polysialylation whereas PST71 and PST62 did not (Figure 15). Previous work demonstrated that wild-type PST is capable of binding to NCAM [314]. To determine if the ability

of a PST mutant protein to compete with endogenous SW2 cell PST and reduce cell surface NCAM polysialylation was specifically due to the ability of a PST mutant to bind to NCAM, I utilized a co-immunoprecipitation approach (Figures 16 and 17). I found that the ability of a PST mutant protein to compete with endogenous PST correlated directly with that protein's ability to bind to NCAM. In total, my data indicate the residues 71-127 of PST contain amino acid sequences and residues that are critical for the recognition of NCAM. Furthermore, the ability of these sequences to compete is due to their ability to mediate NCAM binding.

Previous work in the laboratory suggested that autopolysialylated polySTs might more efficiently polysialylate NCAM [310]. However, in support of the laboratory hypothesis that the initial enzyme-substrate interaction is mediated by protein-protein contacts, our co-immunoprecipitation studies suggest that the interaction between the identified region in PST and NCAM is mediated directly through amino acid contacts, and does not involve polySia chains on PST N-glycans (autopolysialylation). This can be seen in two different instances within my co-immunoprecipitation data. First, PST H331K, PST140 and sPST140 demonstrate a similar level of NCAM binding as fully autopolysialylated wild-type PST (Figure 16), even though these three PST mutants are not autopolysialylated due to their lack of catalytic activity (see Figure 12). Secondly, my co-immunoprecipitation studies utilizing PST140, PST127, PST71 and PST62 were performed in Lec2 CHO cells, which lack the CMP-sialic acid transporter and hence cannot synthesize polySia (Figure 17). In these studies, wild-type PST was still capable of binding to NCAM, and PST140 and PST127 also were able to bind similarly. Since these results indicate a protein-protein interaction, I can conclude that the loss of NCAM recognition and binding when residues 71-127 in PST are lost is due to the loss of a particular sequence within this PST region that mediates these functions.

In some cases, a lower level of expression of truncated PST proteins was noticed in the course of the experiments. First, I could not detect any PST105 expression. For this reason, I could not say at this point in my studies if the sequences involved in NCAM recognition were specifically confined to the PBR. Secondary structure prediction programs such as PSIPRE [327] predict the region around PST residue 105 to be in a random coil formation, suggesting that no crucial secondary structure was interrupted. However, it is possible that this particular PST mutant was not folding properly early on in the ER, hence it was degraded early and not detected in our studies.

Secondly, a common theme was seen in our competition studies when utilizing the truncated PST proteins was that a fewer number of SW2 cells expressed a particular truncation mutant, compared to full-length PST proteins. Since our truncations were quite drastic, eliminating over sixty percent of the wild-type PST enzyme, it is possible that these truncated PST mutants are subject to degradation at a higher rate than PST. However, SW2 cells that did express our PST140, sPST140 and PST127 truncated proteins exhibited the ability to compete with endogenous PST similarly to our full-length mutant (PST H331K), suggesting that our PST truncated mutants, where expressed, are capable of blocking the function of endogenous PST. On a similar note, the expression levels of PST127, PST71 and PST62 were lower than those of the wild type enzyme in our co-immunoprecipitation studies. Nonetheless, the ability of PST127 to bind to NCAM was still comparable to that of PST and PST140 (Figure 17), even though these latter two demonstrated a higher level of expression. Furthermore, the expression level of PST71 is similar to that of PST127, but the former does not bind to NCAM, suggesting that the lack of binding to NCAM seen in our co-immunoprecipitation work is not due to a reduction in PST mutant expression. It should be noted that as seen earlier, all of our mutant PST proteins that express are capable of trafficking to the Golgi just like wild-type PST, suggesting that there is no drastic alterations in protein folding or trafficking.

In summary, I have identified the region within PST encompassing residues 71-127 as a region critical for the recognition of NCAM. This result is consistent with previous NCAM polysialylation studies from our laboratory and others, described earlier. However, this region is still quite broad, and the main question now becomes the following: what are the specific amino acid residues within this region that are involved in NCAM recognition? Focusing on the polyST PBR region, I next sought to identify specific amino acid residues within our identified region that mediate the PST-NCAM protein-protein interaction.

CHAPTER IV

MUTATIONAL ANALYSIS OF ST8SIA IV RESIDUES ARG^{82} AND ARG^{93} INDICATE THAT THEY ARE NECESSARY FOR NCAM RECOGNITION AND SUGGESTS A ROLE FOR THE PST POLYBASIC REGION IN THE POLYSIALYLATION OF MULTIPLE SUBSTRATES

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Introduction

Previous work in our laboratory suggested an important role for the PST polybasic region (PBR) in NCAM polysialylation [305]. The PST PBR was given its name because it consisted of a stretch of 35 amino acids encompassing residues 71-105 that contained a series of seven basic residues, including three arginines (Arg^{82}, Arg^{87}, and Arg^{93}) and four lysines (Lys^{72}, Lys^{83}, Lys^{99} and Lys^{103}) (Figure 18). Replacement of Arg^{82} and Arg^{93} singly or doubly with alanines reduced or eliminated the polysialylation of NCAM, respectively. Furthermore, each of these mutants reduced NCAM polysialylation without an equal decrease in PST autopolysialylation, suggesting that these residues specifically function for the recognition of the NCAM substrate. Since my goal was to identify specific amino acid residues involved in NCAM recognition, and Arg^{82} and Arg^{93} fall within our identified region that is critical for recognition (residues 71-127), I decided to begin my pursuit by investigating if the ability of these two PBR residues to affect NCAM polysialylation is specifically due to their ability to mediate NCAM recognition.

Experimental Approach

To determine the significance of PST residues Arg^{82} and Arg^{93} specifically for NCAM recognition, I created a series of PST point mutants for utilization in competition studies as described previously. Since our initial focus is on Arg^{82} and Arg^{93} in PST, we first mutated these residues singly or doubly to alanine (R82A and R93A) in a PST mutant, PST H331K, that was previously shown to successfully compete with endogenous PST and reduce SW2 cell NCAM polysialylation. The premise of this approach is that if the resultant PST R82A and/or R93A mutant is no longer able to successfully compete with wild-type enzyme and reduce NCAM polysialylation, as will be determined with OL.28-anti polySia antibody staining of NCAM, then Arg^{82} and/or Arg^{93} would be deemed critical for the original PST mutant's ability to compete, and hence critical for NCAM recognition.

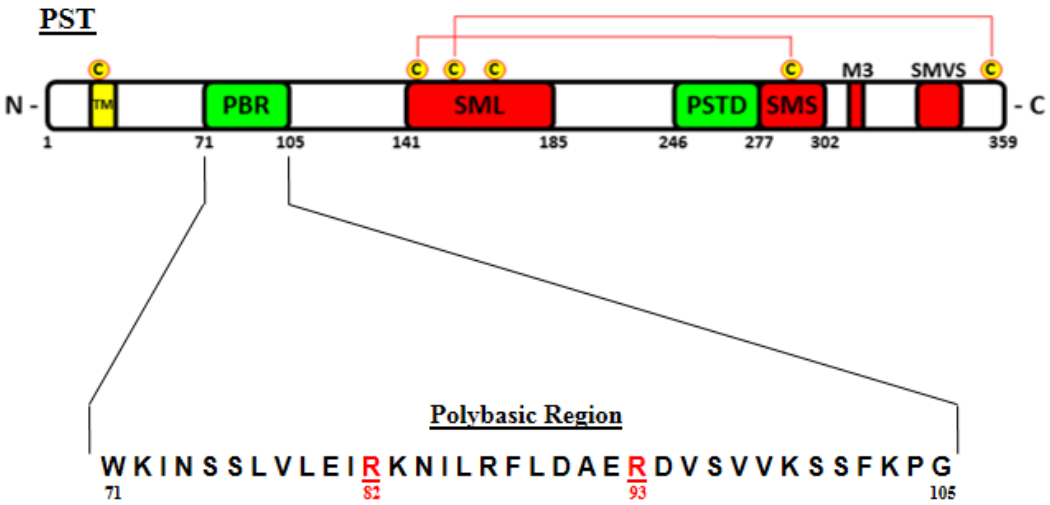

Figure 18. Sequence of the PST PBR and the location of Arg^{82} and Arg^{93}. The Polybasic Region (PBR) of PST consists of thirty-five amino acid residues located between the transmembrane region and the large sialylmotif of PST. It consists of seven basic residues, including three arginine and four lysine residues. The two arginine residues that are the center of our focus here are highlighted in red.

I first attempted to make the R82A and R93A mutations singly and double in PST140 and PST127, since these two truncated PST mutants have already shown to be able to competitively inhibit NCAM polysialylation in SW2 cells. However, I found that replacing these residues in both PST140 and PST127 compromised their folding, leading to some retention in the endoplasmic reticulum. Therefore, I decided to focus on the impact of replacing these residues on the ability of PST H331K to block SW2 cell NCAM polysialylation. I created the R82A and R93A mutations both singly and doubly in PST H331K, as shown in Figure 19 (PST H331K R82A, PST H331K R93A and PST H331K R82A/R93A). As a control, I also evaluated the impact of expressing a catalytically active PST R82A/R93A double mutant on SW2 cell NCAM polysialylation. Since expressing exogenous wild-type PST increased SW2 cell surface polysialylation above basal levels, it was expected that this effect would be lost in the presence of PST R82A/R93A.

Results

All PST and PST H331K PBR point mutant proteins created localize to the Golgi apparatus

As done previously, all created PST and PST H331K PBR point mutant proteins were examined for their ability to localize to the Golgi apparatus. Here, Myc-tagged PST R82A/R93A, PST H331K R82A, PST H331K R93A, and PST H331K R82A/R93A were individually expressed in COS-1 cells and protein localization was determined by staining the cells with anti-Myc epitope tag antibody (Figure 20). Similar to PST and PST H331K (Figure 12), all created PST mutants were predominately localized to the Golgi apparatus and were thus used for competition studies.

Arg^{82} and Arg^{93} in the PST PBR are required for the ability of PST H331K to block NCAM polysialylation

PST, PST H331K, and their respective PBR mutant proteins were expressed individually in SW2 cells as described previously and the cells were stained with anti-Myc epitope and OL.28 anti-polySia antibodies to identify cells expressing our exogenous PST mutants and cell surface NCAM polysialylation, respectively (Figure 21). As shown previously, expressing wild-type PST enhanced the polysialylation of NCAM and allowed detection of the autopolysialylated enzyme in the Golgi. However, SW2 cells expressing the PST R82A/R93A mutant exhibited no

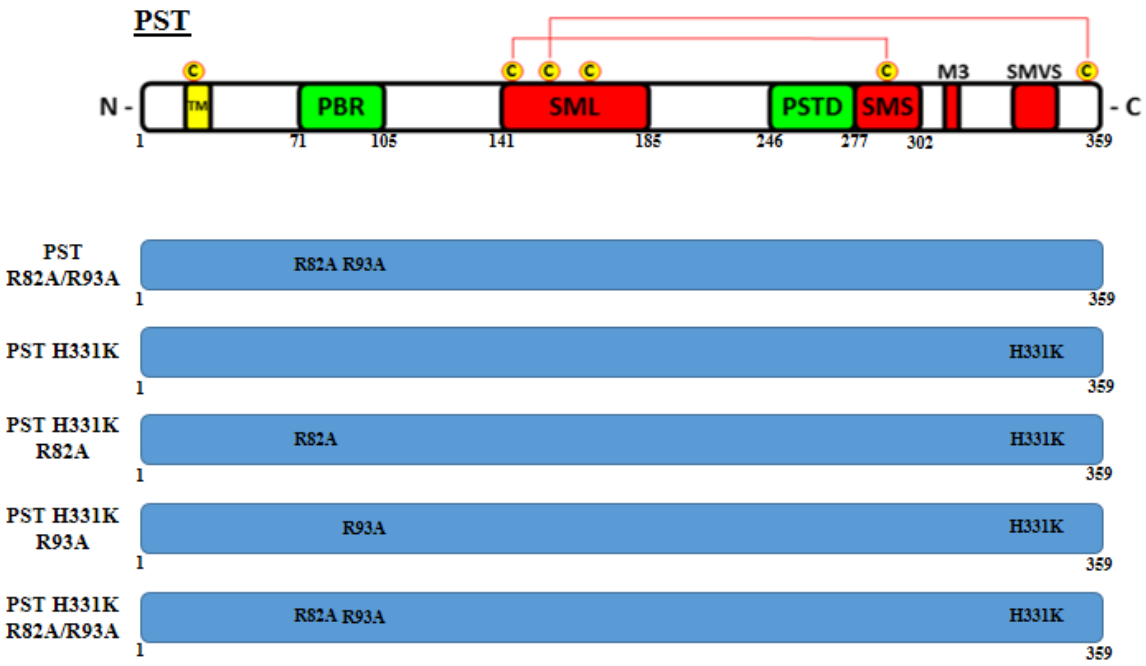

Figure 19. Schematic of PST and PST H331K PBR mutants created. Arg^{82} and Arg^{93} were mutated to alanine either singly or doubly within the PST H331K protein to determine if this protein is still capable of competing with endogenous PST and reducing NCAM polysialylation. Both Arg^{82} and Arg^{93} were also mutated together to alanines in wild-type PST to determine if the raise in SW2 cell surface polysialylation seen with expression of exogenous PST is compromised by mutation of these two residues. All PST proteins created contain a Myc epitope tag attached to their C-terminus.

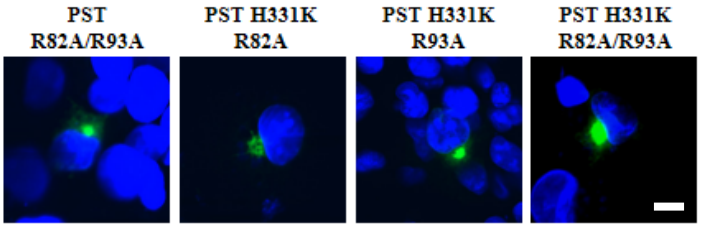

Figure 20. All PST and PST H331K PBR mutant proteins localize to the Golgi apparatus. Myc-tagged PST R82A/R93A, PST H331K R82A, PST H331K R93A, or PST H331K R82A/R93A were expressed in COS-1 cells as described in Chapter II. Cells were then washed, fixed with methanol, and subjected to staining with anti-Myc antibody (*green*) to determine protein localization. DAPI staining was utilized to indicate the location of the nucleus (*blue*). Scale bar = 10 μm.

These data were previously published in: Zapater, J.L., and Colley K.J. (2012) Sequences Prior to Conserved Catalytic Motifs of Polysialyltransferase ST8Sia IV Are Required for Substrate Recognition. *J. Biol. Chem.* 287:6441-53.

enhanced cell surface polysialylation above basal levels, even though I was able to detect this enzyme's autopolysialylation in the Golgi (Figure 21, *PST R82A/R93A*). This result is consistent with the inability of the PST R82A/R93A mutant to recognize endogenous SW2 cell NCAM. What was even more striking though, whereas PST H331K significantly decreased NCAM polysialylation in SW2 cells, the introduction of the R82A/R93A mutation appeared to abolish the ability of PST H331K to compete with the endogenous wild-type PST enzyme, suggesting that both Arg^{82} and Arg^{93} are necessary for the competitive effect seen with PST H331K (Figure 21, *PST H331K R82A/R93A*). Introduction of single point mutants, PST H331K R82A and PST H331K R93A, into SW2 cells also demonstrated that these proteins are unable to effectively compete with endogenous enzyme (Figure 21, *PST H331K R82A* and *PST H331K R93A*). However, whether there is a difference in the extent to which the competitive effect is lost between these single mutants and PST H331K R82A/R93A double mutant is difficult to discern in this particular competition assay since the level of cell surface NCAM polysialylation is measured qualitatively. It is also difficult to discern if one of the residues is more critical for competition than the other. From these results, I concluded that Arg^{82} and Arg^{93} within the PST PBR are important for PST H331K to compete with endogenous enzyme and reduce NCAM polysialylation, hence making these residues important for NCAM recognition. However, to quantify the extent to which Arg^{82} and Arg^{93} are individually responsible for recognition, I performed a modified competition assay in COS-1 cells.

Effective NCAM recognition requires the combined efforts of Arg^{82} and Arg^{93}

To more precisely quantify the contribution of Arg^{82} and Arg^{93} for NCAM recognition, I wanted to create an assay that was more sensitive at measuring changes in NCAM polysialylation and that was quantitative. I first attempted to stay with an SW2 cell model by trying to generate SW2 cells that would stably express the various PST constructs. Unfortunately, I found that these SW2 cell lines were difficult to establish and that the selection procedure for stable clones (utilizing Geneticin), unnaturally increased the levels of SW2 cell polysialylation. Therefore, in lieu of this, I established a triple expression system in COS-1 cells in which NCAM and PST would be co-expressed with an excess of a PST competitor protein, such as PST H331K. For this assay, I expressed V5-tagged NCAM, Myc-tagged PST, and either a Myc-tagged PST H331K or a PST H331K PBR mutant in COS-1

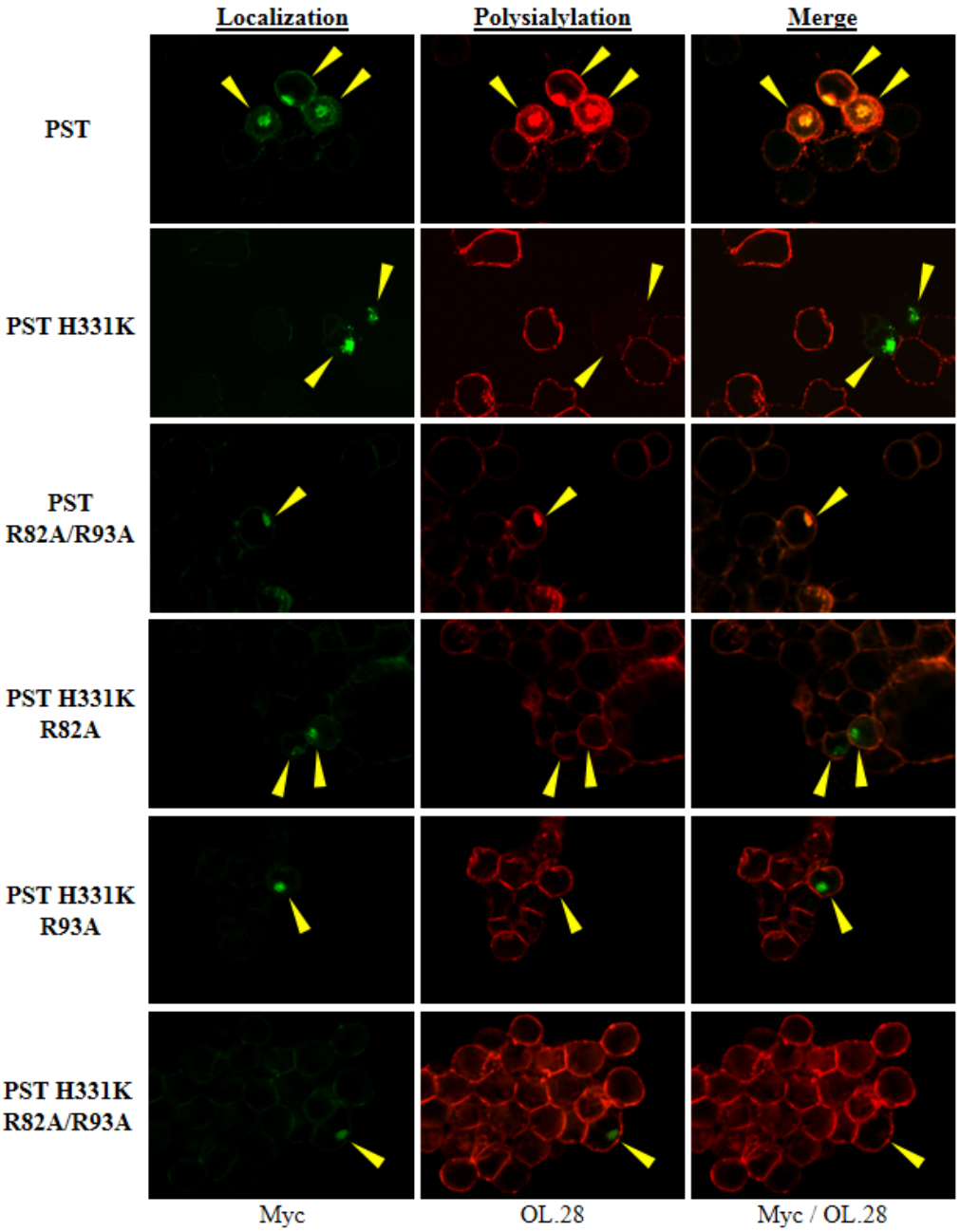

Figure 21. Replacing Arg^{82} and Arg^{93} blocks the ability of PST to enhance the polysialylation of SW2 cell NCAM and the ability of PST H331K to inhibit the polysialylation of SW2 cell NCAM by endogenous PST. Myc-tagged PST, PST R82A/R93A, PST H331K, PST H331K R82A, PST H331K R93A, and PST H331K R82A/R93A were transiently expressed in SW2 cells. Eighteen hours post-transfection, cells were fixed and indirect immunofluorescence was performed to analyze enzyme localization (*Green, localization*) or total polysialylation (*Red, polysialylation*). Cells expressing exogenous PST and PST mutant proteins are marked with yellow arrowheads. Cells were examined using a Zeiss 200 M inverted confocal microscope, 63X oil immersion objective. Scale bar = 10 μm.

These data were previously published in: Zapater, J.L., and Colley K.J. (2012) Sequences Prior to Conserved Catalytic Motifs of Polysialyltransferase ST8Sia IV Are Required for Substrate Recognition. *J. Biol. Chem.* 287:6441-53.

cells at a ratio of 1:1:6 (NCAM:PST:PST competitor). NCAM was precipitated from cell lysates with anti-V5 antibody, and the level of NCAM polysialylation was assessed by immunoblotting with the OL.28 anti-polySia antibody (Figure 22, *upper panel*). Relative levels of NCAM expression (Figure 22, *middle panel*) as well as overall enzyme expression (Figure 22, *lower panel*), were evaluated by immunoblotting a small sample of lysate with anti-V5 and anti-Myc epitope tag antibodies, respectively. Furthermore, using NIH ImageJ, I quantified the relative NCAM polysialylation observed in the presence of PST H331K and its mutants in the representative immunoblot shown.

We found that in the presence of PST and no competitor, NCAM was polysialylated (Figure 22, *lane 1*). However, NCAM polysialylation was undetectable when excess PST H331K was co-expressed with wild-type PST (Figure 22, *lane 2*). Expression of PST H331K with either the R82A or R93A mutation decreased but did not eliminate the ability of PST H331K to block NCAM polysialylation, as 42% and 31% of NCAM polysialylation by wild-type PST was retained, respectively (Figure 22, *lanes 3 and 4*). The partial competition seen here correlates with the reduction but not elimination in NCAM polysialylation seen when these residues are mutated singly in PST [291]. However, when both Arg^{82} and Arg^{93} were mutated to alanines in PST H331K, the ability of this protein to block NCAM polysialylation was eliminated (Figure 22, *lane 5*), correlating with an elimination in NCAM polysialylation seen when these residues are doubly mutated in PST [291]. These results indicate that the PST H331K double mutant is unable to compete with wild-type PST, and strongly suggests that PST residues Arg^{82} and Arg^{93} are together important for NCAM polysialylation because they are mediating PST-NCAM recognition.

<u>The polysialylation of NCAM, SynCAM 1, and NRP-2 by PST exhibits different requirements for PBR residues Arg^{82} and Arg^{93}</u>

Since the process of substrate polysialylation is considered to be highly protein-specific, our finding that Arg^{82} and Arg^{93} in the PST PBR are required for the recognition and polysialylation of NCAM made us curious as to the significance of these residues for the polysialylation of other substrates. Are these residues specifically used to recognize NCAM and NCAM only? Or does substrate specificity in the polysialylation process result from the ability of these two residues to recognize the small subset of polysialylated glycoproteins? To answer these

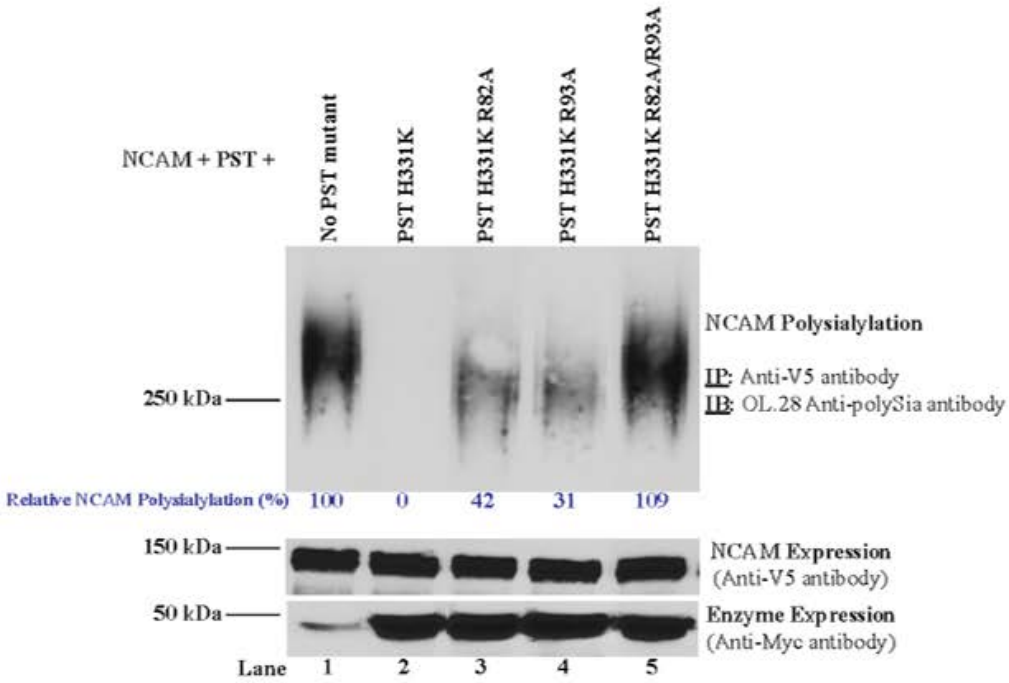

Figure 22. The ability of PST H331K to inhibit the polysialylation of NCAM by endogenous SW2 cell PST is eliminated when both Arg^{82} and Arg^{93} are replaced with alanine residues. V5-tagged NCAM and Myc-tagged PST were expressed in COS-1 cells without (*lane 1*) or with Myc-tagged PST H331K, PST H331K R82A, PST H331K R93A, or PST H331K R82A/R93A in a 1:1:6 ratio (*lanes 2-5*). Eighteen hours post-transfection, cells were lysed and NCAM was immunoprecipitated using anti-V5 antibody. Immunoprecipitated NCAM was subjected to SDS-PAGE and immunoblotting to analyze NCAM polysialylation (*top panel*). Relative NCAM, PST and PST mutant protein expression levels were assessed by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE and immunoblotting with anti-V5 antibody (*NCAM Expression, middle panel*) or anti-Myc antibody (*Enzyme Expression, bottom panel*). Note that "Enzyme Expression" reflects the expression levels of both wild type and mutant PST proteins.

These data were previously published in: Zapater, J.L., and Colley K.J. (2012) Sequences Prior to Conserved Catalytic Motifs of Polysialyltransferase ST8Sia IV Are Required for Substrate Recognition. *J. Biol. Chem.* 287:6441-53.

questions, we decided to expand our studies to examine the significance of PST residues Arg^{82} and Arg^{93} for the polysialylation of synaptic cell adhesion molecule 1 (SynCAM 1) and neuropilin-2 (NRP-2). These two substrates were chosen due to their significance in human physiology and a growing body of recent evidence suggesting alterations in their function upon polySia modification. The roles of SynCAM 1 and NRP-2 in mammalian physiology have been previously discussed. PolySia addition to SynCAM 1 has been shown to reduce SynCAM 1 homophilic interactions, thus regulating the formation of a neuron-glial synapse [289]. PolySia addition to NRP-2 occurs on O-linked glycans, and its role is still a matter of debate. It has been suggested that polySia on NRP-2 inhibits dendritic cell-induced T cell activation and proliferation [293] and enhances dendritic cell migration through stimulation of CCR7 receptor signaling [294].

To determine the significance of Arg^{82} and Arg^{93} for the overall polysialylation of SynCAM 1 and NRP-2, we created the R82A or R93A mutations singly or doubly in PST. Following co-expression of V5-tagged NCAM, SynCAM 1 or NRP-2 with Myc-tagged PST, PST R82A, PST R93A, or PST R82A/R93A in COS-1 cells, the substrate proteins were precipitated with anti-V5 epitope antibody and their polysialylation was analyzed as described earlier (Figures 23-25, *upper panels*). Relative substrate expression (Figures 23-25, *middle panels*) and enzyme expression levels (Figures 23-25, *lower panels*) were also assayed as previously described. As seen previously, replacing Arg^{82} and Arg^{93} with alanine residues decreased the polysialylation of NCAM (Ref. 291 and Figure 23, *lanes 1-3*). Furthermore, a significant decrease in NCAM polysialylation required that both Arg^{82} and Arg^{93} be replaced (Figure 23, *lane 4*), confirming previous results [305]. In contrast, mutation of Arg^{82} to alanine alone or in combination with Arg^{93}, eliminates SynCAM 1 and NRP-2 polysialylation by PST (Figure 24 and 25, *lanes 1, 2, and 4*). However, mutation of Arg^{93} alone to alanine did not reduce SynCAM 1 or NRP-2 polysialylation (Figure 24 and 25, *lane 3*). Taken together, these results suggested that there are different requirements for specific PST PBR residues for the polysialylation of different substrates. Combined with data presented above, these results suggest that the PST PBR functions specifically in the recognition of substrates for polysialylation, and that Arg^{82} in the PST PBR may be required for general substrate recognition, whereas Arg^{93} specifically aids the recognition of NCAM.

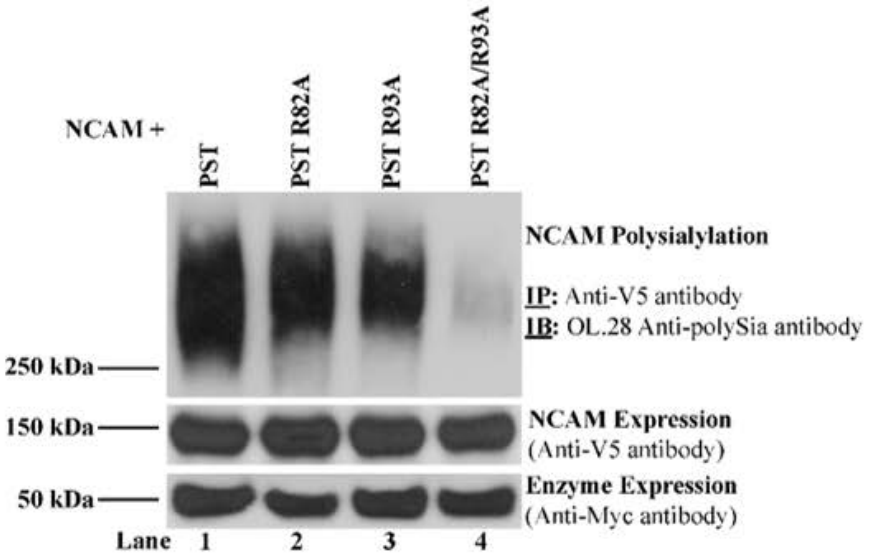

Figure 23. The ability of PST to effectively polysialylate NCAM requires the combined efforts of Arg^{82} and Arg^{93}. NCAM-V5 was co-expressed with Myc-tagged PST, PST R82A, PST R93A, or PST R82A/R93A in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of NCAM polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

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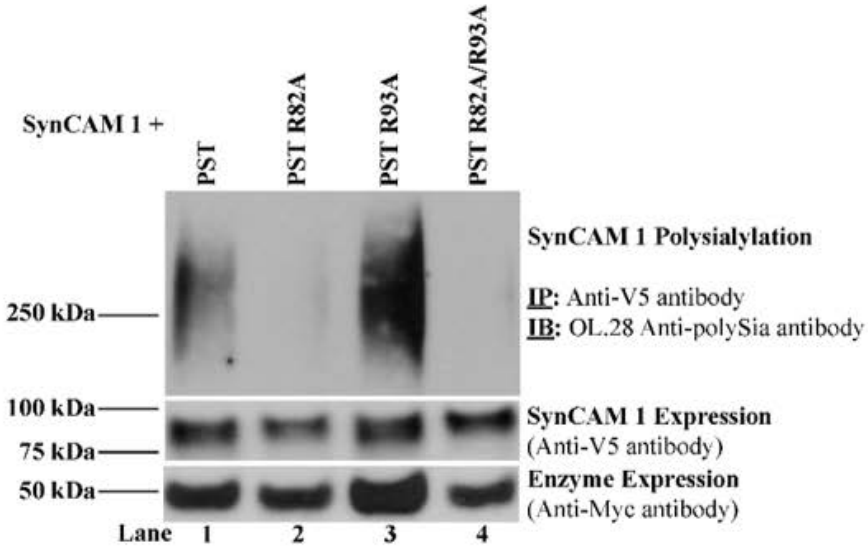

Figure 24. The ability of PST to effectively polysialylate SynCAM 1 requires Arg^{82}, but not Arg^{93}. SynCAM 1-V5 was co-expressed with Myc-tagged PST, PST R82A, PST R93A, or PST R82A/R93A in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of SynCAM 1 polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

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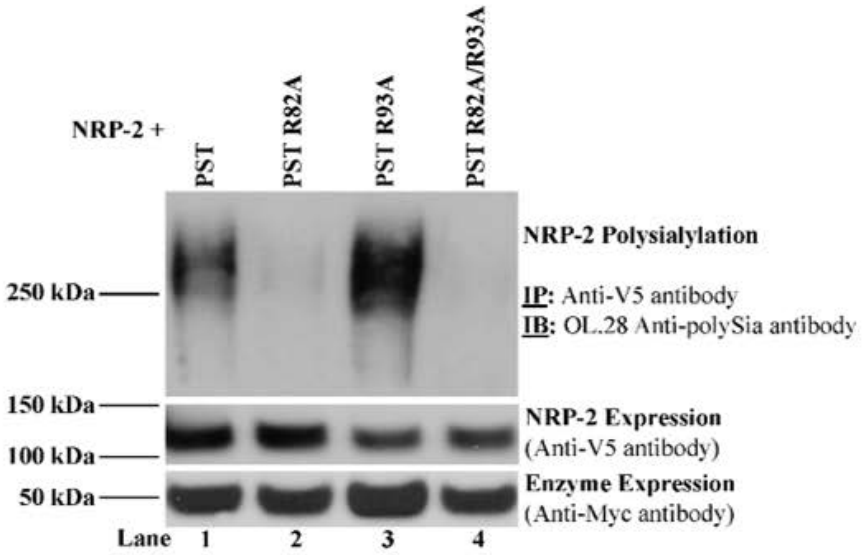

Figure 25. Similar to SynCAM 1, the ability of PST to effectively polysialylate NRP-2 requires Arg^{82} only. NRP-2-V5 was co-expressed with Myc-tagged PST, PST R82A, PST R93A, or PST R82A/R93A in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of NRP-2 polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

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Discussion

Previous work from our laboratory suggested that the PST PBR, as well as Arg^{82} and Arg^{93} within this region, are important for the effective polysialylation of NCAM [305]. My competition and binding studies presented earlier demonstrated that PST sequences between residues 71 and 127 are required for NCAM recognition (Figures 14-17). Since PST residues Arg^{82} and Arg^{93} fall within our identified region and are required for NCAM polysialylation, I sought to determine if these are important because they specifically mediate NCAM recognition. Here, I found that replacing these two arginine residues in the catalytically-inactive PST H331K protein eliminated its ability to compete with endogenous PST in SW2 cells (Figure 21). Furthermore, the triple expression competition experiment performed in COS-1 cells indicated that both residues individually make substantial contributions to NCAM recognition and polysialylation, with Arg^{93} possibly playing a slightly larger role (Figure 22). The replacement of both arginine residues together completely eliminated the ability of PST H331K to compete with wild-type PST, suggesting that these are the two critical residues in PST that mediate the recognition of NCAM.

I continued the analysis of PST residues Arg^{82} and Arg^{93} by determining if they are also critical for the polysialylation of SynCAM 1 and NRP-2. To date, the function of the polyST PBR for polysialylation of substrates other than NCAM has not been pursued. I found that PST residue Arg^{82} plays a predominant role in SynCAM 1 and NRP-2 polysialylation (Figures 24 and 25). However, replacing Arg^{93} does not have an impact on the proper polysialylation of SynCAM 1 and NRP-2, despite it have a critical role for NCAM polysialylation (Figures 23-25). Altogether, these results emphasized the role of Arg^{82} and Arg^{93} in substrate recognition and polysialylation rather than general enzyme activity, and raised the intriguing possibility that different substrates may be recognized by slightly different binding surfaces of the polySTs.

In terms of NCAM recognition, Arg^{82} and Arg^{93} in the PST PBR seem to be the two residues that completely mediate the PST-NCAM interaction. How is it possible that just two arginine residues could have such a large impact on NCAM recognition? Researchers studying the propensity of certain amino acids at protein interfaces suggest that these interfaces favor residues that can engage in hydrophobic and electrostatic interactions, such as tryptophan, arginine and tyrosine [reviewed in 328]. Arginine has been found to be preferred in “hot spots” of

binding energy because it is capable of multiple types of favorable interactions, including forming up to five hydrogen bonds as well as a salt bridge utilizing the positive charge on its guanidinium moiety [329]. In fact, an arginine-aspartate salt bridge is found at the interface of the α- and β-subunits of the $GABA_A$ receptor, and removal of this bridge through alanine mutagenesis compromises GABA binding and subunit association [330]. Applying these observations to the PST-NCAM interaction, Arg^{82} and Arg^{93} may promote recognition through the formation of hydrogen bonds and salt bridges with residues in NCAM. In fact, previous results from our laboratory suggest that it is plausible that salt bridges could be formed between PST and NCAM. These earlier studies identified an acidic patch on the surface of NCAM FN1 that is composed of aspartic acid and glutamic acid residues [312]. When these surface residues were mutated together to alanines, there was a significant reduction in NCAM polysialylation by PST [312]. Furthermore, there was a reduction in the physical binding of PST to NCAM [314]. These data indicate that the NCAM FN1 surface acidic patch may form part of a docking site for PST, with Arg^{82} and Arg^{93} in PST serving at the complementary sequences that recognize the docking site.

Since replacement of Arg^{82} and Arg^{93} with alanines prevented PST H331K from successfully competing with endogenous PST in SW2 cells or with wild-type PST in COS-1 cells, we sought to demonstrate a role for these arginine residues in a direct PST-NCAM protein-protein interaction using co-immunoprecipitation experiments. However, these experiments proved to be unsuccessful. We were unable to detect a reduction in PST-NCAM binding when Arg^{82} and Arg^{93} were replaced with alanines, despite the profound effect this alteration has on NCAM polysialylation. One possibility stemming from recent results from our laboratory is that there may be a secondary PST-NCAM interaction potentially utilizing residues in the NCAM Ig5 domain [316]. Since the Ig5 domain is the location of polySia addition, it is possible that PST residues mediating such an interaction may be located in regions outside of our area of focus (residues 71-127), such as within the catalytic sialylmotifs or possibly the polysialyltransferase domain (PSTD), which similar to the PBR contains many arginine and lysine residues. In this respect, when we tested the binding of truncated PST mutants to NCAM, it is possible that these PST proteins did not contain the amino acids that are involved in a secondary interaction at NCAM Ig5. Therefore, utilizing truncated PST mutants, we were able to demonstrate with co-immunoprecipitation the significance of one PST region (the

PBR) on NCAM binding. However, when we mutate specific amino acids within residues 71-127 in PST H331K, the entire PST sequence is still present in this protein, and other interactions with NCAM may still be able to occur. A search for more PST residues within the catalytic region of PST would be warranted. A second possibility is that the polySTs can interact with substrates in more than one way, but only when they are aligned properly with the NCAM N-glycans to be modified can substrate polysialylation take place. In support of this idea, our laboratory demonstrated that deleting NCAM FN1 eliminates polyST binding [314], however, replacing NCAM FN1 with other FN1 domains allows binding, but not polysialylation [318]. This suggests that residues in NCAM FN1 allow the precise positioning of enzyme and substrate and that this is critical for substrate polysialylation.

In light of the above discussion concerning the positioning of enzyme and substrate, another notable point is that replacing Arg^{82} and Arg^{93} had different effects on polysialylation of the three substrates tested. These results suggest that the polySTs use different but overlapping sequences to recognize their substrates and position them appropriately for polysialylation. Removal of specific PST basic residues may redirect substrates to other polyST surfaces that do not allow optimal alignment of enzyme and substrate, and consequently prevent polysialylation without greatly altering enzyme-substrate binding. Related to this possibility, previous work in our laboratory demonstrated that changes in the linker region between NCAM Ig5 and FN1 or in the FN1 sequences at the Ig5-FN1 interface are capable of eliminating NCAM polysialylation, thus highlighting the importance of a defined Ig5-FN1 relationship for the ability of a polyST interacting with the NCAM FN1 domain to access and polysialylate the Ig5 N-glycans [318].

A particularly interesting and repeatable observation was the consistent increase in SynCAM 1 and NRP-2 polysialylation observed when PST Arg^{93} is replaced by alanine (Figures 24 and 25). One possibility is that the presence of Arg^{93} forms a less optimal binding site for these substrates. If such is the case, then eliminating Arg^{93} could enhance polysialylation of SynCAM 1 and NRP-2 by optimizing their interaction with other basic residues in the polyST PBR. However, the increase in SynCAM 1 and NRP-2 polysialylation noted is not drastically increased above polysialylation by wild-type PST and this observation does not detract from the fact that the polysialylation of these substrates was eliminated when PST Arg^{82} was mutated to alanine.

In this chapter, I analyzed the importance of two PST PBR residues for the recognition of NCAM and for the overall polysialylation of SynCAM 1 and NRP-2. My findings here—that Arg^{82} and Arg^{93} are differentially required for the polysialylation of different substrates—was quite intriguing because it raised new questions concerning the polyST PBR region. In addition to Arg^{82} and Arg^{93}, the PST PBR contains five other basic residues—are any of these other basic residues critical for SynCAM 1 and NRP-2 polysialylation? Furthermore, the complimentary PBR in STX contains eight basic residues. Compared with the PST PBR, are the analogous STX PBR basic residues also important for NCAM, SynCAM 1 and NRP-2 polysialylation? Or, are non-analogous, unique combinations of basic residues required for each polyST-substrate combination? Furthermore, I have seen that PST PBR residues that are important for NCAM polysialylation are critical because they mediate NCAM recognition. Does this scenario hold true for STX PBR basic residues that may be important for NCAM polysialylation? What about for PST-SynCAM 1, PST-NRP-2, STX-SynCAM 1 and STX-NRP-2 recognition? To get a more comprehensive understanding of the polyST PBR in recognition of multiple substrates, I will now introduce STX into my studies, and examine all basic residues in both the PST and STX PBRs individually to determine their significance in the polysialylation and recognition of NCAM, SynCAM 1 and NRP-2.

CHAPTER V

DIFFERENT COMBINATIONS OF OVERLAPPING ST8SIA IV/PST POLYBASIC REGION BASIC RESIDUES ARE REQUIRED FOR EFFECTIVE RECOGNITION AND POLYSIALYLATION OF NCAM, SYNCAM 1, AND NRP-2

Portions of this chapter will be submitted to The Journal of Biological Chemistry

Introduction

The differential requirements of PST PBR residues Arg^{82} and Arg^{93} for the polysialylation of different substrates raises the question as to whether there are more amino acids that are required to work in tandem with the above arginine residues that have not yet been identified. Furthermore, the differential requirement of Arg^{93} for substrate polysialylation as seen in Chapter IV raises to possibility that the pattern of PBR basic residues critical for recognition and polysialylation of NCAM, SynCAM 1 and NRP-2 may be specific to each substrate. To shed more light on these points, and to continue my pursuit of identifying specific amino acids that are required for the biochemical mechanism of substrate polysialylation, I expanded my studies in this chapter by analyzing the significance of each PST PBR basic residue individually for the polysialylation and recognition of NCAM, SynCAM 1, and NRP-2.

Experimental approach

I first sought to determine the significance of each PST PBR basic residue for substrate polysialylation. For this, I mutated each basic residue in each PBR to alanine singly (Figure 26), expressed each polyST mutant with NCAM, SynCAM 1, or NRP-2, and measured resultant substrate polysialylation. To determine the role of each polyST PBR basic residue for substrate recognition, I made the same mutations in the PST H331K background, and utilized these polyST mutants in competition studies utilizing the COS-1 triple expression system as in Chapter IV.

Results

<u>All PST PBR mutants localize to the Golgi and are autopolysialylated</u>

Myc-tagged wild-type PST and each PST PBR mutant were expressed individually in COS-1 cells, which were then stained with anti-Myc antibody to examine the cellular localization of these proteins (Figure 27,

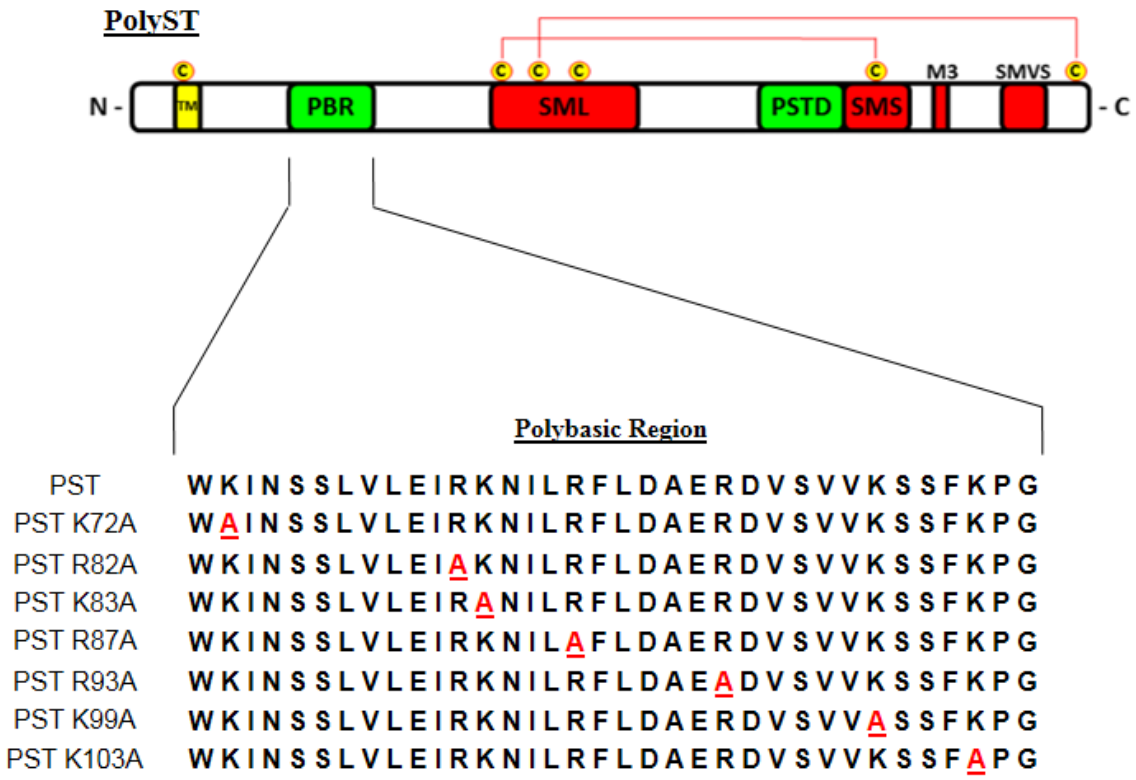

Figure 26. Creation of PST PBR alanine mutants. Each arginine and lysine residue in the PST PBR was individually mutated to alanine, leading to the creation of each of the above constructs. The location of each mutation in each PST construct is marked with an underlined red “A”.

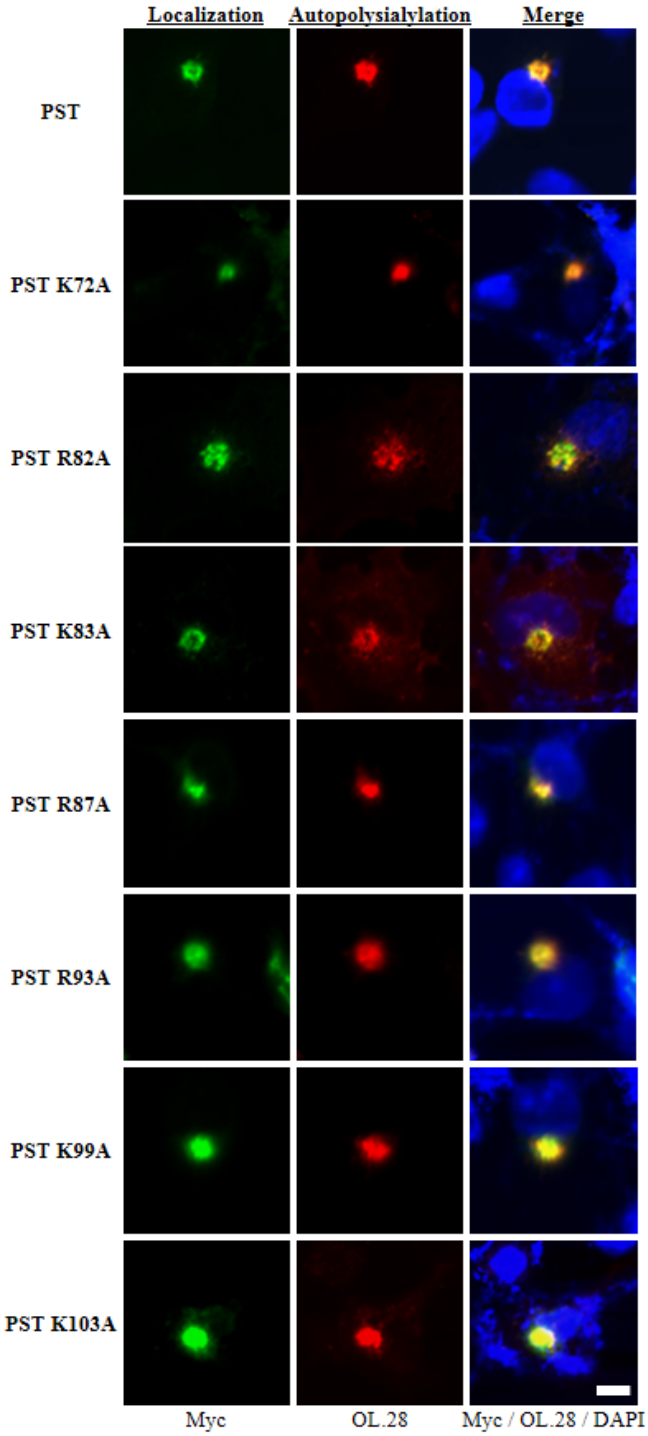

Figure 27. All PST PBR mutants localize to the Golgi and are autopolysialylated comparably to wild-type enzyme. Myc-tagged PST and PST PBR mutants were expressed in COS-1 cells, then fixed with methanol and stained with anti-Myc (*Localization, green*) and OL.28 anti-polySia antibodies (*Autopolysialylation, red*). DAPI staining indicates the location of the nucleus (*Localization and Merge, blue*). Scale bar = 10 μm.

Localization). To examine enzyme autopolysialylation, the expressing COS-1 cells were also stained with OL.28 anti-polySia antibody (Figure 27, *Autopolysialylation*). I found that like wild-type PST, all of the PST PBR mutants were expressed and localized primarily to the Golgi apparatus. Also, all of these mutant proteins were autopolysialylated, suggesting that these proteins still retain their catalytic function.

The polysialylation of NCAM, SynCAM 1 and NRP-2 by PST requires different sets of PBR basic residues

To determine the significance of specific PST PBR basic residues for NCAM, SynCAM 1, and NRP-2 polysialylation, Myc-tagged PST or a PST PBR mutant was co-expressed with V5-tagged NCAM, SynCAM 1, or NRP-2 in COS-1 cells. The V5-tagged substrates were precipitated from cell lysates with anti-V5 antibody, and the level of substrate polysialylation was assessed by immunoblotting with OL.28 anti-polySia antibody (Figures 28-30, *top panels*). Relative levels of substrate expression (Figures 28-30, *middle panels*) as well as overall enzyme expression (Figures 28-30, *bottom panels*), were evaluated by immunoblotting a small sample of lysate with anti-V5 and anti-Myc epitope tag antibodies, respectively. Using NIH ImageJ, I quantified the relative amount of substrate polysialylation observed in the presence of each PST PBR mutant.

As a confirmation of previous results from Foley *et al.* [305] and results from Chapter IV, NCAM polysialylation was drastically reduced in the presence of PST proteins containing the R82A and R93A mutations (Figure 28, *top panel, lanes 4 and 7*), as compared to wild-type PST (Figure 28, *top panel, lane 2*). Although smaller alterations in NCAM polysialylation are seen with other PST PBR mutants, Arg^{82} and Arg^{93} are the main PBR basic residues that are critical for polySia addition to NCAM by PST. In contrast, SynCAM 1 polysialylation by PST was disrupted by the R82A, R82A, R87A and K99A mutations (Figure 29, *top panel, lanes 4, 6, and 8*), but not the R93A mutation (Figure 29, *top panel, lane 7*). Furthermore, NRP-2 polysialylation was reduced in the presence of only the PST R82A or K99A mutations (Figure 30, *top panel, lanes 4 and 8*). In the case of NRP-2, Arg^{82} was found to reduce NRP-2 polysialylation slightly more than Lys^{99} (82% compared to 65% for the latter residue). From these results, I saw that PST residue Arg^{82} is important for the polysialylation of all three substrates, whereas Arg^{87} and Arg^{93} specifically aid SynCAM 1 and NCAM polysialylation, respectively, and Lys^{99} is required for effective

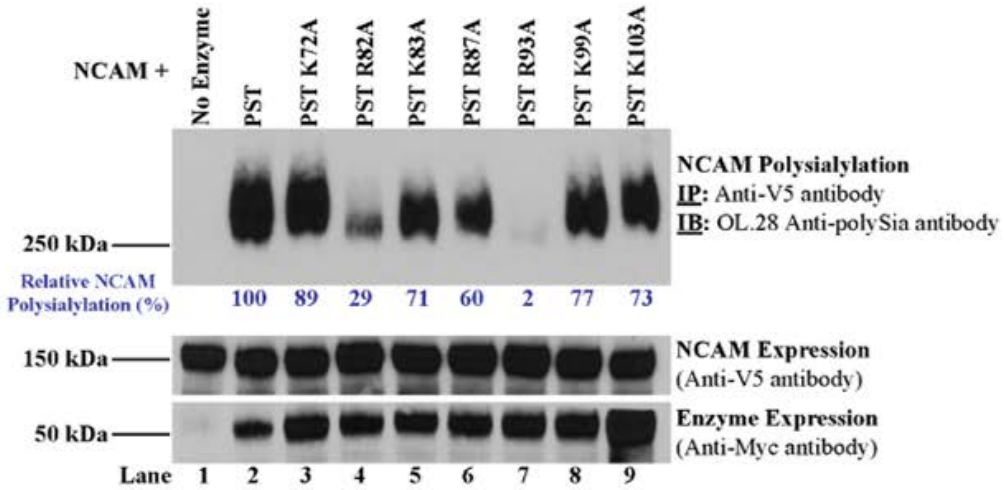

Figure 28. Individual mutations of each PST PBR basic residue to alanine suggests that the combined efforts of Arg^{82} and Arg^{93} are most relevant for effective NCAM polysialylation. NCAM-V5 was co-expressed without (*lane 1*) or with Myc-tagged PST, or a PST PBR mutant (*lanes 2-9*) in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of NCAM polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

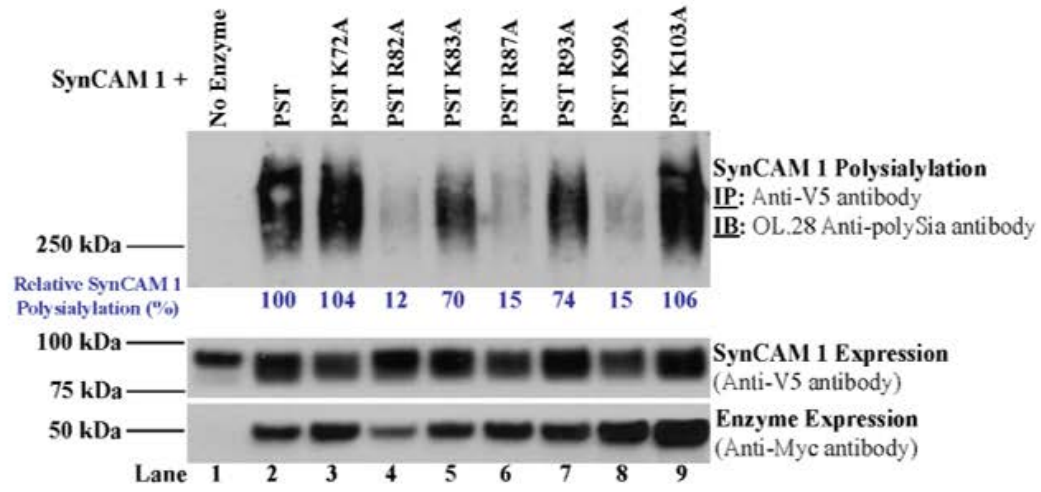

Figure 29. The ability of PST to effectively polysialylate SynCAM 1 requires Arg^{82}, Arg^{87}, and Lys^{99} within the PBR region. SynCAM 1-V5 was co-expressed without (*lane 1*) or with Myc-tagged PST, or a PST PBR mutant (*lanes 2-9*) in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of SynCAM 1 polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

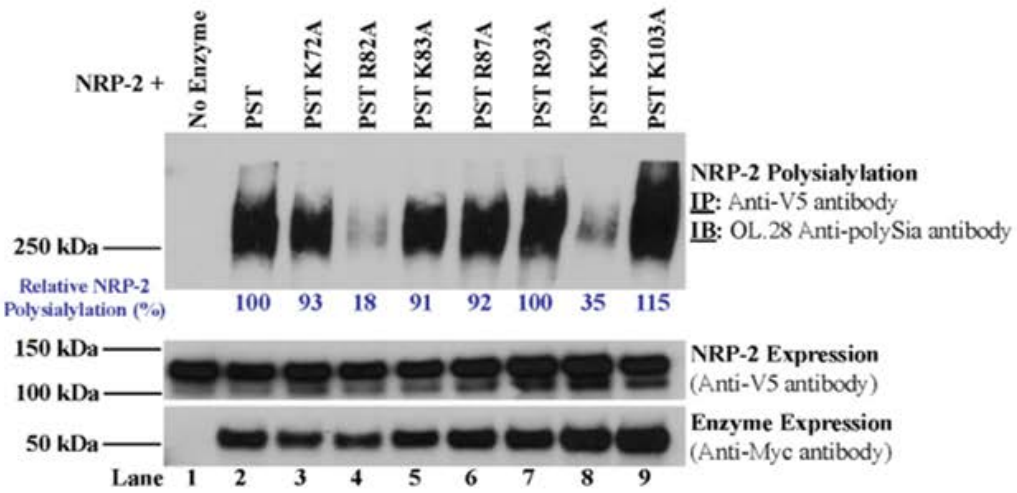

Figure 30. The ability of PST to effectively polysialylate NRP-2 requires Arg^{82} and Lys^{99} within the PBR region. NRP-2-V5 was co-expressed without (*lane 1*) or with Myc-tagged PST, or a PST PBR mutant (*lanes 2-9*) in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of NRP-2 polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

polysialylation of both SynCAM 1 and NRP-2. I now sought to determine the significance of these residues for substrate recognition.

Localization of PST H331K and PST H331K PBR alanine mutants

To examine the effect of each PST PBR basic residue on the recognition of NCAM, SynCAM 1 and NRP-2, I first created each PBR alanine mutation displayed in Figure 26 within the PST H331K protein. Each of these Myc-tagged proteins was first expressed in COS-1 cells and examined for proper cellular localization by fixing the cells with methanol, followed by staining with anti-Myc epitope tag antibody (Figure 31). I found that like wild-type PST and PST H331K (Figure 31, *top row, left and middle images*), PST H331K proteins with the K72A, R82A, R87A, R93A, K99A, or K103A mutations all were localized predominantly to the Golgi. However, PST H331K K83A (Figure 31, *middle row, middle image*) was localized to reticular structures reminiscent of the endoplasmic reticulum. This suggested that PST H331K K83A is not trafficking properly, possibly due to protein misfolding.

PST PBR basic residues that are critical for NCAM, SynCAM 1, and NRP-2 polysialylation are involved in polyST-substrate recognition

Utilizing a triple expression system in COS-1 cells for my competition studies, I expressed V5-tagged NCAM, SynCAM 1 or NRP-2 with either Myc-tagged PST alone or with a Myc-tagged PST H331K or PST H331K PBR mutant in COS-1 cells at a ratio of 1:1:6 (NCAM:PST:PST H331K competitor) (Figures 32-34). The V5-tagged substrates were precipitated from cell lysates with anti-V5 antibody, and their polysialylation was assessed by immunoblotting with the commercially available 12F8 anti-polySia antibody (Figures 32-34, *top panels*). Relative levels of substrate expression (Figures 32-34, *middle panels*), as well as overall enzyme expression (Figures 32-34, *bottom panels*), were evaluated by immunoblotting a small sample of lysate with anti-V5 and anti-Myc epitope tag antibodies, respectively. As before, NIH ImageJ was used to quantify the relative substrate polysialylation observed in the presence of PST H331K and its corresponding PBR mutants.

As shown earlier, in the presence of an excess of PST H331K, NCAM polysialylation by wild-type PST is eliminated (Figure 32, *top panel, lane 2*). Also, the presence of excess PST H331K eliminated the ability of PST to

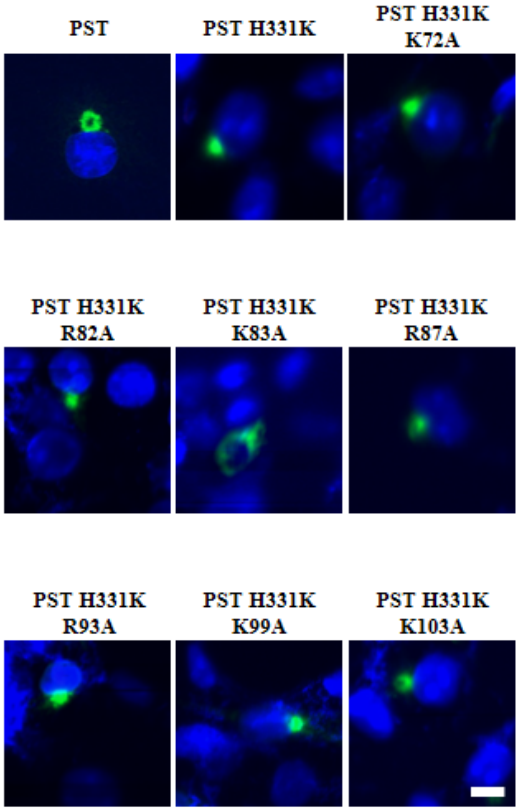

Figure 31. All PST H331K PBR mutant proteins localize to the Golgi apparatus with the exception of PST H331K K83A. Myc-tagged PST, PST H331K, or a PST H331K PBR mutant were expressed in COS-1 cells. Cells were then washed, fixed with methanol, and subjected to staining with anti-Myc antibody (*green*) to determine protein localization. DAPI staining was utilized to indicate the location of the nucleus (*blue*). Scale bar = 10 μm.

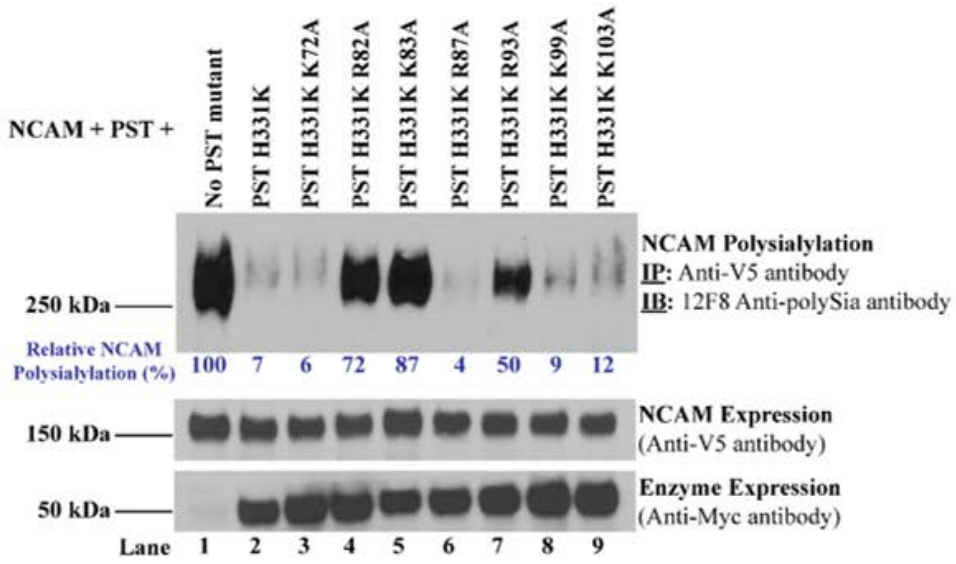

Figure 32. The ability of PST H331K to inhibit the polysialylation of NCAM by wild-type PST is compromised when Arg^{82} and Arg^{93} are replaced with alanine residues. V5-tagged NCAM and Myc-tagged PST were expressed in COS-1 cells without (*lane 1*) or with Myc-tagged PST H331K, or a PST H331K PBR alanine mutant in a 1:1:6 ratio (*lanes 2-9*). Eighteen hours post-transfection, cells were lysed and NCAM was immunoprecipitated using anti-V5 antibody. Immunoprecipitated NCAM was subjected to SDS-PAGE and immunoblotting with 12F8 anti-polySia antibody to analyze NCAM polysialylation (*top panel*). Relative NCAM, PST and PST mutant protein expression levels were assessed by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE and immunoblotting with anti-V5 antibody (*NCAM Expression, middle panel*) or anti-Myc antibody (*Enzyme Expression, bottom panel*). Note that "Enzyme Expression" reflects the expression levels of both wild type and mutant PST proteins.

polysialylate both SynCAM 1 and NRP-2 (Figures 33 and 34, *top panels, lane 2*). Among the PST H331K PBR alanine mutants, I found that the ability of PST H331K to compete with wild-type PST to reduce NCAM polysialylation was significantly decreased in the presence of the R82A, K83A or R93A mutations (Figure 32, *top panel, lanes 4, 5 and 7*), as a significant increase in NCAM polysialylation was observed in the presence of these competitors. As mentioned before, PST H331K K83A is predominantly localized to the endoplasmic reticulum, possibly because it is misfolded. Consequently, it is not surprising that this mutant is unable to compete with the wild type Golgi localized PST. However, at the same time I cannot definitively determine the significance of this PST PBR residue for substrate recognition. Compared to the ability of PST H331K to compete with wild-type enzyme (Figure 32, *top panel, lane 2*), competition was found to be less in the R93A mutant (43%, compared to PST H331K) than in the R82A mutant (65%, compared to PST H331K), which might suggest differences in these residues' overall binding affinity. This hypothesis would still need to be further tested. However, this result is still consistent with our finding in Figure 22 in the previous chapter, in which the R82A PST H331K mutant was a slightly better competitor than the R93A mutant.

The ability of PST H331K to competitively inhibit the polysialylation of SynCAM 1 appeared to be reduced in the presence of the R82A, R87A, or K99A mutations (Figure 33, *top panel, lanes 4, 6, and 8*). Notably, these are the same residues that are important for SynCAM 1 polysialylation by PST as shown in Figure 29. The PST H331K K83A mutant was unable to serve as a competitive inhibitor, but again this is likely to due to its mislocalization (Figure 33, *top panel, lane 5*). Although 12F8 anti-polySia staining appeared to be weak, it appeared that PST Arg^{82}, Arg^{87}, and Lys^{99} contribute nearly equally to SynCAM 1 recognition.

Analysis of the PST PBR residues involved in NRP-2 recognition demonstrated that the presence of the R82A or K99A mutations in PST H331K significantly reduced this protein's ability to compete with wild-type PST to block NRP-2 polysialylation (Figure 34, *top panel, lanes 4 and 8*). As with the NCAM studies, where the R93A mutation of the PST H331K competitor led to a lower recovery of NCAM polysialylation relative to the R82A mutation, the K99A mutation in PST H331K also lead to a lower recovery of NRP-2 polysialylation relative to the

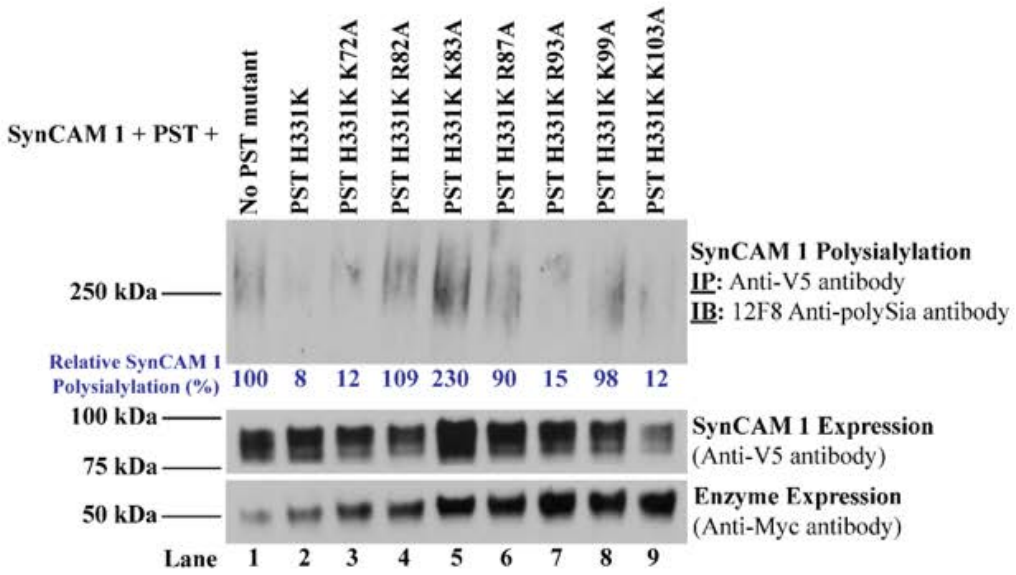

Figure 33. The ability of PST H331K to inhibit the polysialylation of SynCAM 1 by wild-type PST is compromised when Arg^{82}, Arg^{87}, and Lys^{99} are replaced with alanine residues. V5-tagged SynCAM 1 and Myc-tagged PST were expressed in COS-1 cells without (*lane 1*) or with Myc-tagged PST H331K, or a PST H331K PBR alanine mutant in a 1:1:6 ratio (*lanes 2-9*). Eighteen hours post-transfection, cells were lysed and SynCAM 1 was immunoprecipitated using anti-V5 antibody. Immunoprecipitated SynCAM 1 was subjected to SDS-PAGE and immunoblotting with the 12F8 anti-polySia antibody to analyze SynCAM 1 polysialylation (*top panel*). Relative SynCAM 1, PST and PST mutant protein expression levels were assessed by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE and immunoblotting with anti-V5 antibody (*SynCAM 1 Expression, middle panel*) or anti-Myc antibody (*Enzyme Expression, bottom panel*). Note that "Enzyme Expression" reflects the expression levels of both wild type and mutant PST proteins.

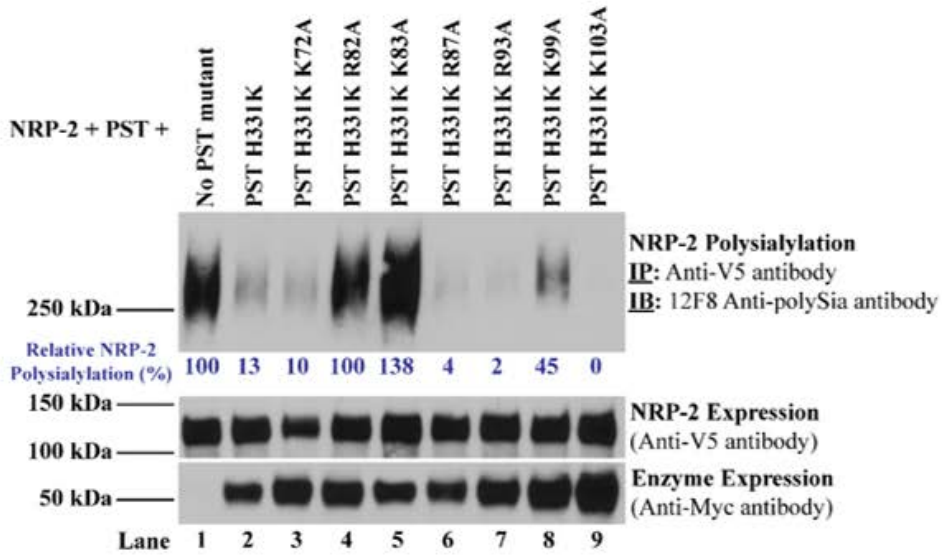

Figure 34. The ability of PST H331K to inhibit the polysialylation of NRP-2 by wild-type PST is compromised when Arg^{82} and Lys^{99} are replaced with alanine residues. V5-tagged NRP-2 and Myc-tagged PST were expressed in COS-1 cells without (*lane 1*) or with Myc-tagged PST H331K, or a PST H331K PBR alanine mutant in a 1:1:6 ratio (*lanes 2-9*). Eighteen hours post-transfection, cells were lysed and NRP-2 was immunoprecipitated using anti-V5 antibody. Immunoprecipitated NRP-2 was subjected to SDS-PAGE and immunoblotting with 12F8 anti-polySia antibody to analyze NRP-2 polysialylation (*top panel*). Relative NRP-2, PST and PST mutant protein expression levels were assessed by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE and immunoblotting with anti-V5 antibody (*NRP-2 Expression, middle panel*) or anti-Myc antibody (*Enzyme Expression, bottom panel*). Note that "Enzyme Expression" reflects the expression levels of both wild type and mutant PST proteins.

R82A mutation. From these data, it appears that Arg^{82} plays a predominant role in NCAM and NRP-2 recognition, with Arg^{93} and Lys^{99}, playing secondary roles for NCAM and NRP-2 recognition, respectively.

In sum, these results suggest that the PST PBR residues that are critical for substrate polysialylation are the same ones that are critical for substrate recognition. These results further support the role of the PST PBR in substrate recognition, and indicate that overlapping sets of basic residues within the PBR of PST are responsible for NCAM, SynCAM 1, and NRP-2 recognition. A summary of the key PST PBR residues that are involved in substrate recognition and polysialylation are listed below in Table XII, with the residues aligned for comparison purposes.

TABLE XII: SUMMARY OF PST PBR RESIDUES REQUIRED FOR EFFECTIVE SUBSTRATE RECOGNITION AND POLYSIALYLATION

Substrate	**Enzyme**	**PBR Basic Residues Critical for Polysialylation**
NCAM	**PST**	Arg^{82} Arg^{93}
SynCAM 1	**PST**	Arg^{82} Arg^{87} Lys^{99}
NRP-2	**PST**	Arg^{82} Lys^{99}

Discussion

In this chapter, I expanded my investigations by evaluating the polysialylation of SynCAM 1 and NRP-2 alongside NCAM, and examined the role of every PST and STX PBR basic residue in the recognition and polysialylation of these substrates. We found that the polysialylation of NCAM, SynCAM 1, and NRP-2 by PST all required PBR residue Arg^{82}, which suggests that this arginine residue plays a general role in substrate

polysialylation (Figures 28-30). Furthermore, PST Arg^{87} and Arg^{93} were only required for polysialylation of SynCAM 1 and NCAM, respectively, suggesting that these residues specifically aid the polysialylation of these two substrates (Figures 28 and 29). Finally, Lys^{99} was required for effective polysialylation of SynCAM 1 and NRP-2 (Figures 29 and 30). Our competition analyses demonstrated that the residues identified as important for the polysialylation of a specific substrate were the same residues that were important for the recognition of that substrate, as their mutation in PST H331K to alanine significantly reduced the ability of this catalytically-inactive PST mutant to compete with wild-type PST and reduce that substrate polysialylation, suggesting that PST H331K was no longer able to effectively recognize NCAM, SynCAM 1, or NRP-2 (Figures 32-34). These data suggest that the identified PST PBR basic residues above are critical for polysialylation because they are required for the recognition of NCAM, SynCAM 1, and NRP-2. Additionally, the combinations of critical PST PBR basic residues differed by the substrate. In all, these data indicate that the PST PBR is indeed involved in substrate recognition, and that different sets of overlapping basic residues within are utilized to recognize each substrate.

Although we identified the same PST PBR residues as being critical for both recognition and overall polysialylation in each PST-substrate combination, it is interesting to note that in some cases the ability of a specific PST PBR basic residue to compete does not necessarily match its ability to affect overall polysialylation (Table XIII).

TABLE XIII: COMPARISON OF REDUCED POLYSIALYLATION AND COMPETITION LEVELS OBSERVED FOR TWO SPECIFIC PST PBR ALANINE MUTANTS

		Substrate Polysialylation	**Competition**		
Substrate	**PST H331K PBR Mutant**	**Percent Polysialylation Lost**	**Percent Polysialylation w/ PST H331K**	**Percent Polysialylation w/ PST H331K PBR Mutant**	**Percent Competition Lost**
NCAM	R93A	**98**	7	50	**43**
NRP-2	K99A	**65**	13	45	**32**

As seen in Table XIII, the R93A mutation in PST reduces the polysialylation of NCAM by 98% compared to NCAM polysialylation by wild-type PST (Figure 28, *top panel*, *lanes 2 and 7*), but the same mutation in PST H331K only reduces competition by 43% as compared to the amount of competition lost in the presence of PST H331K (Figure 32, *top panel*, *lanes 2 and 7*; Table XIII, "Percent Polysialylation Lost" = "Percent Polysialylation w/ PST H331K PBR mutant" minus "Percent Polysialylation w/ PST H331K"). In Table XIII, the percentages calculated are based on densitometry measurements using NIH ImageJ software and are listed below the top panels in all polysialylation and competition figures in this chapter. Also in Table XIII, for competition studies, the percent of competition lost is being read as the increase in substrate polysialylation by PST observed in the presence of a particular PST H331K PBR mutant as compared substrate polysialylation observed in the presence of PST H331K, which serves as a template and baseline measurement for competition. Secondly, the K99A mutation in PST reduces the polysialylation of NRP-2 by 65% (Figure 34, *top panel*, *lane 8*), but the same mutation in PST H331K only reduces competition by 32% as compared to PST H331K (Figure 38, *top panel*, *lanes 2 and 8*).

Why does the decrease in polysialylation of a particular PBR residue mutant not match its ability to reverse competition by the inactive enzyme? A possibility that would need to be tested is that the overall binding affinity of a polyST depends on the sum of the interaction of several residues, but some residues may contribute more to overall binding than others. For example, mutating Lys^{99} to alanine in PST H331K may not alter the binding affinity of PST H331K for NRP-2 as much as mutating Arg^{82}. This would correlate with a lower level of competition seen in the presence of the K99A mutation, as it would be more difficult to fully competitively inhibit the binding of the wild-type enzyme to NRP-2. Along these lines, mutating K99A in wild-type PST also may not affect binding affinity as much as the R82A mutation. However, it is possible that effective substrate polysialylation requires a completely stable interaction between PST and NRP-2. As this would no longer exist in the presence of either the R82A or K99A mutations regardless of affinity changes, the PST-substrate interaction may not be capable of lasting for a sufficient amount of time required to synthesize long polySia chains. Why is there no difference in the level of competition seen between PST H331K R82A and PST H331K K99A when it comes to SynCAM (Figure 33)? As mentioned before, the PBR residues that are critical for substrate recognition are unique to the polyST-substrate

pairing, and thus the situation may be different in each case. Further binding studies would be necessary to better quantify the role of each mutation that we created in overall affinity of the polySTs for each substrate.

Two other puzzling results were observed in this study. First, while I was able to easily analyze the changes in SynCAM 1 polysialylation with PST PBR mutants using the OL.28 anti-polySia antibody (Figure 29), evaluating alterations in PST H331K competition in the presence of these mutations was more difficult because of a general reduction in staining intensity with 12F8 anti-polySia antibody (Figure 33). One possibility is that the OL.28 anti-polySia antibody used in the first study is simply more effective than the 12F8 anti-polySia antibody used in the second study. This technical issue in combination with reports that suggest that STX may be more effective at polysialylating SynCAM 1 compared to PST [331] may have led to the detection problems. Secondly, we were unable to analyze was the K83A point mutation in PST H331K, as this mutant was found expressed but predominately localized to the endoplasmic reticulum (Figure 31). What is interesting here is that creating this mutation alone in wild type PST results in a correctly folded, autopolysialylated protein (Figure 27). One possibility is that a long-range interaction may be present that critically involves Lys^{83} and His^{331}, which may cause a disruption in protein stability great enough to cause misfolding when both residues are mutated in tandem. The importance of potential long-range interactions within proteins will be discussed in more detail in the next chapter. The unusual disruption of protein stability seen with only one of the PST H331K point mutations that I created suggests that more concrete structural information will be necessary to determine the basis for the misfolding.

In sum, the results presented in this chapter indicate that the PST PBR is critical for the polysialylation of NCAM, SynCAM 1, and NRP-2 because residues within this region function specifically for the recognition of each substrate. This conclusion is based on the observation that specific residues that were found to be necessary for the polysialylation of a specific substrate were the same residues required for PST H331K to effectively compete with wild-type PST and reduce that substrate's polysialylation. Furthermore, the ability of PST to effectively recognize and polysialylate each of the three substrates required different sets of overlapping basic residues within the PST PBR, which correlates with the hypothesis that the mechanism of protein polysialylation is highly protein specific. Thus far in this work, I have identified a region in PST that is necessary for the polysialylation and

recognition of NCAM, SynCAM 1, and NRP-2, as well as pinpointed specific residues within this region that potentially mediate substrate recognition. However, the varying criteria within PST required for the effective polysialylation of each substrate raises the question as to whether these criteria are comparable in the other human polysialyltransferase, STX. Therefore, in the next chapter, I will detail studies that I performed to identify, analyze, and compare with PST, the role of STX PBR basic residues in substrate polysialylation and recognition.

CHAPTER VI

IDENTIFICATION OF ST8SIA II/STX POLYBASIC REGION RESIDUES THAT ARE REQUIRED FOR EFFECTIVE POLYSIALYLATION AND RECOGNITION OF NCAM AND SYNCAM 1

Portions of this chapter will be submitted to The Journal of Biological Chemistry

Introduction

Although I have thus far focused on PST, and to date there are not as many biochemical studies relating to the mechanism by which STX recognizes and polysialylates substrates, several studies suggest that this mechanism will be just as critical to understand. As mentioned previously, STX is expressed at its peak during embryonic development, indicating that it plays critical roles during mammalian development, especially in the brain [197]. Previous mouse ST8Sia II knockout studies from Angata *et al.* [332] demonstrated the presence of ST8Sia II-specific defects. These included misguidance of infrapyramidal mossy fibers and the formation of ectopic neuronal synapses during development, as well as a higher exploratory drive and reduced behavioral responses to Pavlovian fear conditioning in the adult mouse. Additionally, polySia is abnormally re-expressed in a variety of human tumor cells, and the polyST that is responsible for this polySia expression varies by the tumor. It is true that some tumors preferentially re-express PST, including small cell lung carcinoma, rhabdomyosarcoma, and acute myeloid leukemia [281]. However, in non-small cell lung carcinoma, polySia expression is highly dependent on stage, and relies mainly on the presence and activity level of STX [333]. Furthermore, expression of polySia in neuroblastoma tumor cells is the result of a preferential expression of STX [281]. Therefore, a complete understanding of the biochemical mechanism by which both PST and STX add polySia onto substrates is warranted.

Comparison of PST and STX features

To what extent could the role of the STX PBR in substrate recognition and polysialylation be similar or identical to the PST PBR? I first decided to perform a few comparative analyses of the two polySTs. PST and STX are type II transmembrane Golgi localized glycosyltransferases that each possess a large, small and very small sialylmotif, a motif III, as well as polybasic and polysialyltransferase domains. Aligning their sequences using ClustalW [334] demonstrates exactly how much similarity these two polySTs exhibit (Figure 35, *green shaded*

Figure 35. Sequence alignment of ST8Sia II/STX and ST8Sia IV/PST. The amino acid sequences for PST and STX were aligned using ClustalW sequence alignment program [334]. Based on the sequence alignment, amino acids that are identical in the polySTs are bolded and shaded in green. The locations of the catalytic sialylmotifs are marked with a red bar over the residues. The PBR and PSTD are marked with a green bar over the residues corresponding to these regions. The four cysteine residues in each polyST that contribute to disulfide bridges are labeled with a yellow star overhead.

residues). These polySTs share approximately 58% overall sequence identity with a majority of it occurring in the catalytic sialylmotifs and the PBR and PSTD domains (Table XIV). Furthermore, PST and STX share four cysteine residues that participate in two disulfide bridges (Figure 35, *yellow stars*). These disulfide bridges are critical for polyST catalytic activity [307]. One noticeable difference is the fact that STX is sixteen residues longer than PST. The majority of these residues are between the C-terminus of the transmembrane region and the N-terminus of the PBR (Figure 35). Only a small amount of sequence homology occurs prior to the PBR.

TABLE XIV: PERCENTAGE SEQUENCE IDENTITY IN SELECT PST AND STX REGIONS

Region	Total Number of Residues	Number of Identical Residues	Percent Identity
Large Sialylmotif	45	33	73%
Small Sialylmotif	25	21	84%
Motif III	5	5	100%
Very Small Sialylmotif	7	6	86%
Polybasic Region (PBR)	35	21	60%
PolyST Domain (PSTD)	32	26	81%

The PST and STX PBR region consists of a thirty-five amino acid stretch that spans residues 71-105 in PST and 86-120 in STX (Figure 36). There are seven basic residues within the PST PBR consisting of three arginines (Arg^{82}, Arg^{87}, and Arg^{93}) and four lysines (Lys^{72}, Lys^{83}, Lys^{99}, and Lys^{103}). There are eight basic residues within the STX PBR consisting of three arginines (Arg^{87}, Arg^{95}, and Arg^{97}) and five lysines (Lys^{98}, Lys^{102}, Lys^{108}, Lys^{114}, and

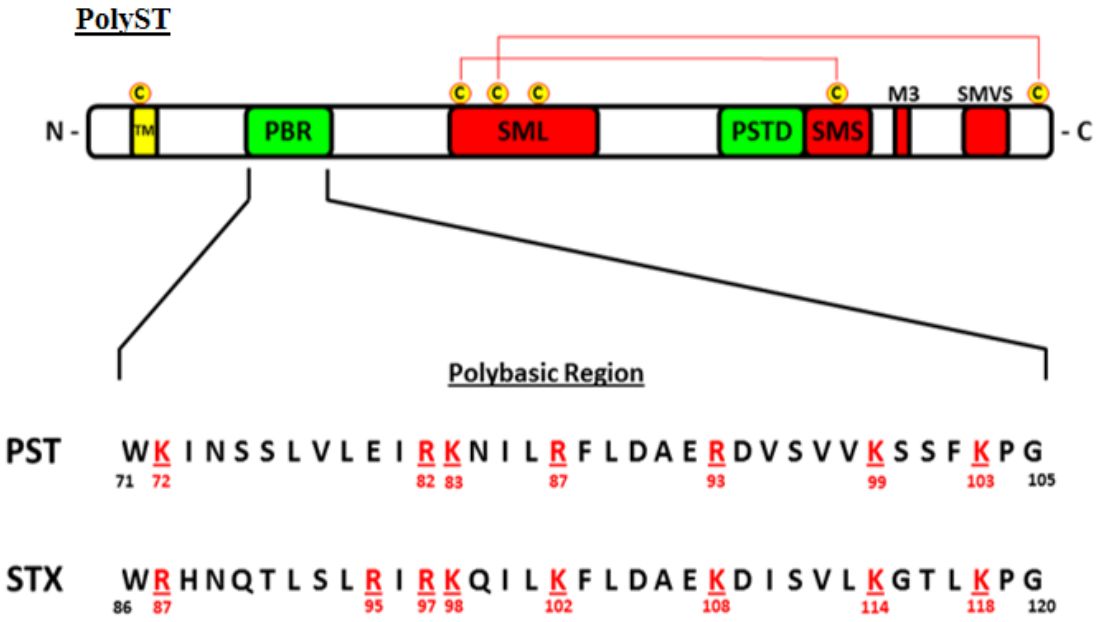

Figure 36. Sequence comparison of the PST and STX PBRs. The PST and STX PBR regions were aligned using Clustal W. The locations of all basic residues within these polyST PBRs are highlighted in red and underlined.

Lys[118]). I could align each PST PBR basic residue with a complimentary basic residue in the STX PBR, with only STX Arg[95] remaining unpaired. It should be noted that my initial hypothesis of the biochemical mechanism underlying substrate recognition and polysialylation by the polySTs was based on experiments with PST and NCAM.

At the time I did these studies, it was still unclear if STX performs polysialylation using the same mechanism as PST. However, our understanding of the significance of select residues within the PST PBR for NCAM recognition and binding, as well as the presence of analogous basic residues in the STX PBR, suggests that the biochemical mechanism of STX-mediated substrate polysialylation may be similar or identical to that of PST. To determine if the mechanism of these two enzymes is the same, I first analyzed the ability of a catalytically-inactive STX protein to recognize and bind to NCAM.

A catalytically-inactive STX protein can compete with wild-type PST to block NCAM polysialylation

To determine if STX contains sequences that are required for recognition of NCAM, one of the initial experiments I did was to determine if a catalytically-inactive STX protein can compete with wild-type PST and inhibit NCAM polysialylation. We created an STX protein in which His[346] was mutated to lysine, creating a full-length catalytically-inactive STX protein (STX H346K). The H346K mutation is analogous to the H331K mutation made in PST, which rendered this polyST inactive. This protein localizes to the Golgi apparatus similarly to wild-type STX (see Figure 40). I set up a triple expression competition assay in COS-1 cells as performed in Chapter IV. V5-tagged NCAM, Myc-tagged PST, and either a Myc-tagged PST H331K, STX H346K, or ST8Sia III was expressed in COS-1 cells at a ratio of 1:1:6 (NCAM:PST:competitor) (Figure 37). ST8Sia III is a related sialyltransferase that autopolysialylates its own N-glycans similarly to PST and STX, but is incapable of polysialylating NCAM, and hence I hypothesized that this protein would not recognize NCAM and would not serve as a competitor [322]. NCAM was precipitated from cell lysates with anti-V5 antibody, and levels of NCAM polysialylation were assessed by immunoblotting with the OL.28 anti-polySia antibody (Figure 37, *top panel*). Relative levels of NCAM expression (Figure 37, *middle panel*) as well as overall enzyme expression (Figure 37, *bottom panel*), were evaluated by immunoblotting a small sample of lysate with anti-V5 and anti-Myc epitope tag

antibodies, respectively. Furthermore, using NIH ImageJ, I quantified the relative NCAM polysialylation observed in the presence of PST H331K, STX H346K, or ST8Sia III in the representative immunoblot shown.

Once again, in the presence of no competitor, NCAM is polysialylated by PST, whereas in the presence of PST H331K, NCAM polysialylation by PST is significantly reduced (Figure 37, *top panel*, *lanes 1-2*). Interestingly, in the presence of STX H346K, NCAM polysialylation by PST is eliminated (Figure 37, *top panel*, *lane 3*), suggesting that STX is capable of recognizing NCAM, and that PST and STX may interact with NCAM at either the same or overlapping sites within NCAM. Overall expression levels of STX H346K were much greater than that of other enzymes, which is typical of the expression levels of all STX proteins in our cellular expression assays (Figure 37, *bottom panel*, *lane 3*). It is unclear if a higher level of STX H346K is required for successful competition, but this does not negate the ability of this protein to compete with wild-type PST for NCAM recognition. ST8Sia III did not prevent NCAM polysialylation by PST (Figure 37, *top panel*, *lane 4*), suggesting that this sialyltransferase's inability to polysialylate NCAM is a result of its inability to recognize NCAM. From this result, we conclude that similar to PST, STX contains sequences that are critical for NCAM recognition and that the NCAM sequences recognized by STX may be identical or overlapping those recognized by PST.

Like PST, STX can bind to NCAM

The results above suggested that like PST, STX should be able to bind to NCAM. I compared the ability of STX and PST and their inactive mutants to bind to NCAM utilizing a co-immunoprecipitation approach as performed in Chapter III. V5-tagged NCAM was co-expressed with Myc-tagged wild-type PST or STX, PST H331K, or STX H346K in COS-1 cells (Figure 38). The enzymes were then immunoprecipitated from the cell lysates with an anti-Myc antibody, and co-immunoprecipitated NCAM-V5 was then detected by immunoblotting with anti-V5 antibody (Figure 38, *top panel*). Relative expression levels of NCAM and enzymes were determined by immunoblotting an aliquot of cell lysate with anti-V5 and anti-Myc antibodies, respectively (Figure 38, *middle* and *bottom* panels).

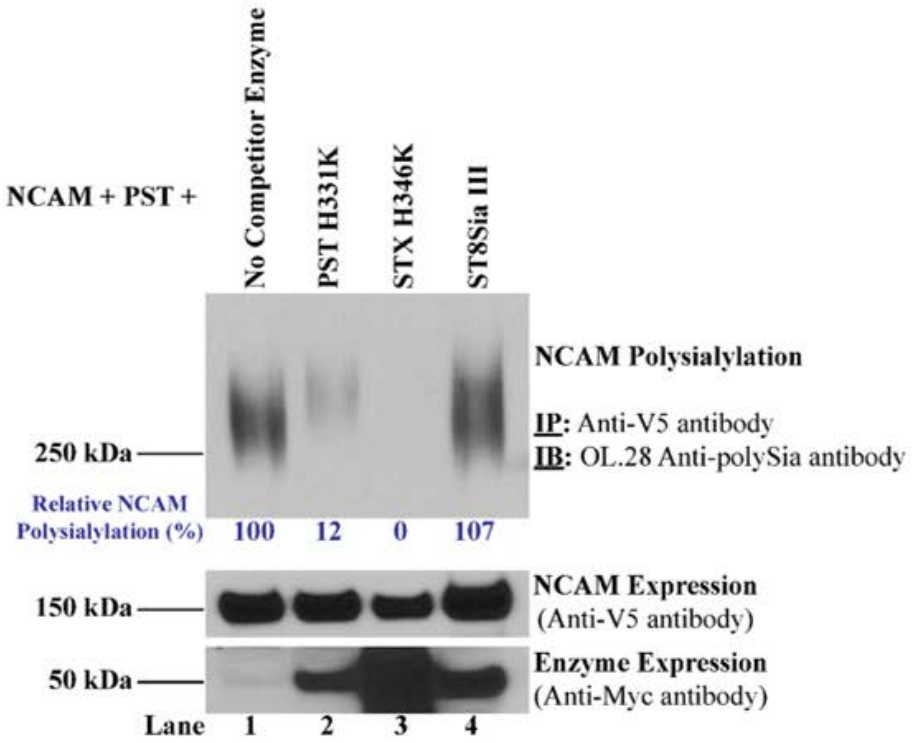

Figure 37. STX H346K cross competes with wild-type PST and reduces NCAM polysialylation. V5-tagged NCAM and Myc-tagged PST were expressed in COS-1 cells without (*lane 1*) or with Myc-tagged PST H331K, STX H346K, or ST8Sia III in a 1:1:6 ratio (*lanes 2-4*). Eighteen hours post-transfection, cells were lysed and NCAM was immunoprecipitated using anti-V5 antibody. Immunoprecipitated NCAM was subjected to SDS-PAGE and immunoblotting to analyze NCAM polysialylation (*top panel*). Relative NCAM, PST and competitor protein expression levels were assessed by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE and immunoblotting with anti-V5 antibody (*NCAM Expression, middle panel*) or anti-Myc antibody (*Enzyme Expression, bottom panel*). Note that "Enzyme Expression" reflects the expression levels of both wild type enzyme and mutant competitor proteins.

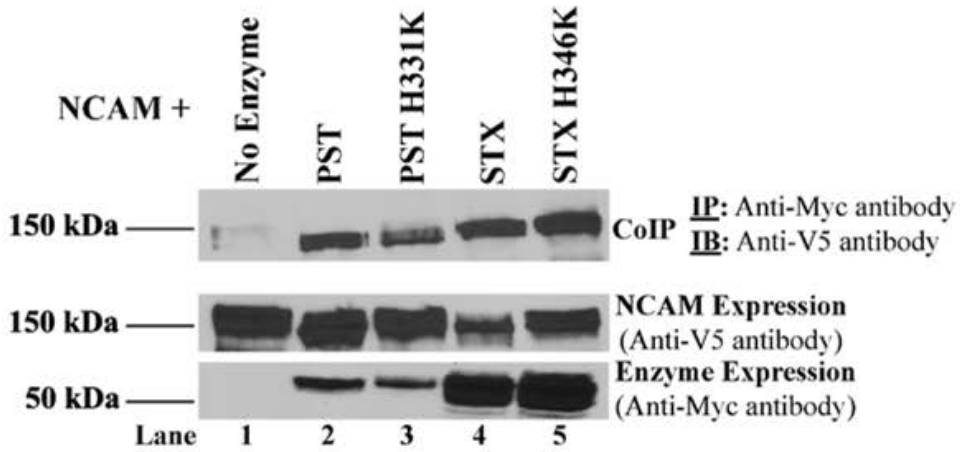

Figure 38. Similar to PST, STX contains sequences that are critical for binding to NCAM. V5-tagged NCAM was co-expressed with Myc-tagged PST, PST H331K, STX, or STX H346K in COS-1 cells. Eighteen hours post-transfection, cells were lysed and lysates were incubated with anti-Myc epitope tag antibody to immunoprecipitate the PST or STX proteins, followed by incubation with protein A-Sepharose beads for one hour. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-V5 antibody to detect co-immunoprecipitated NCAM (*top panel*). To determine the relative expression levels of NCAM and PST proteins, an aliquot of lysate was removed prior to co-immunoprecipitation and subjected to SDS-PAGE and immunoblotting with anti-V5 antibody to detect NCAM (*middle panel*) and anti-Myc antibody to detect the PST or STX proteins (*bottom panel*).

I found that wild type STX and STX H346K bound to NCAM similarly (Figure 38, *top panel*, *lanes 4 and 5*). Furthermore, the ability of STX and STX H346K to bind to NCAM was similar to that of wild type PST and PST H331K (Figure 38, *top panel*, *lanes 2-5*). Taken altogether, like PST, STX contains sequences that are critical for the recognition and binding of the NCAM. Although I did not create truncated STX proteins as I did for PST, based on the above results, I feel confident that the PBR of STX is likely to participate in substrate recognition, and have included a more detailed evaluation of the STX PBR residues in the recognition of various substrates below.

Experimental approach

I first sought to determine the significance of each STX PBR basic residue for substrate polysialylation. For this, I mutated each basic residue in each PBR to alanine singly (Figure 39), expressed each STX mutant protein with NCAM, SynCAM 1, or NRP-2, and measured the resultant polysialylation of each substrate. To determine the role of each STX PBR basic residue for substrate recognition, I made the same mutations in the STX H346K background, and utilized these STX mutants in competition studies utilizing the COS-1 triple expression system as in Chapters IV and V.

Results

STX PBR mutants localize to the Golgi and are autopolysialylated

To first determine if each mutant was localized appropriately and autopolysialylated, Myc-tagged wild-type STX and STX PBR mutants were expressed in COS-1 cells. The cells were then washed, fixed with methanol, and stained with anti-Myc epitope tag antibody (Figure 40, *Localization*) and OL.28 anti-polySia antibody (Figure 40, *Autopolysialylation*). I found that like wild-type STX, all STX PBR alanine mutants were predominately localized to the Golgi and autopolysialylated.

The polysialylation of NCAM and SynCAM 1 by STX requires larger sets of PBR basic residues than PST

To determine the significance of specific STX PBR basic residues for substrate poylsialylation, Myc-tagged STX or a STX PBR mutant was co-expressed with V5-tagged NCAM, SynCAM 1, or NRP-2 in COS-1 cells. The

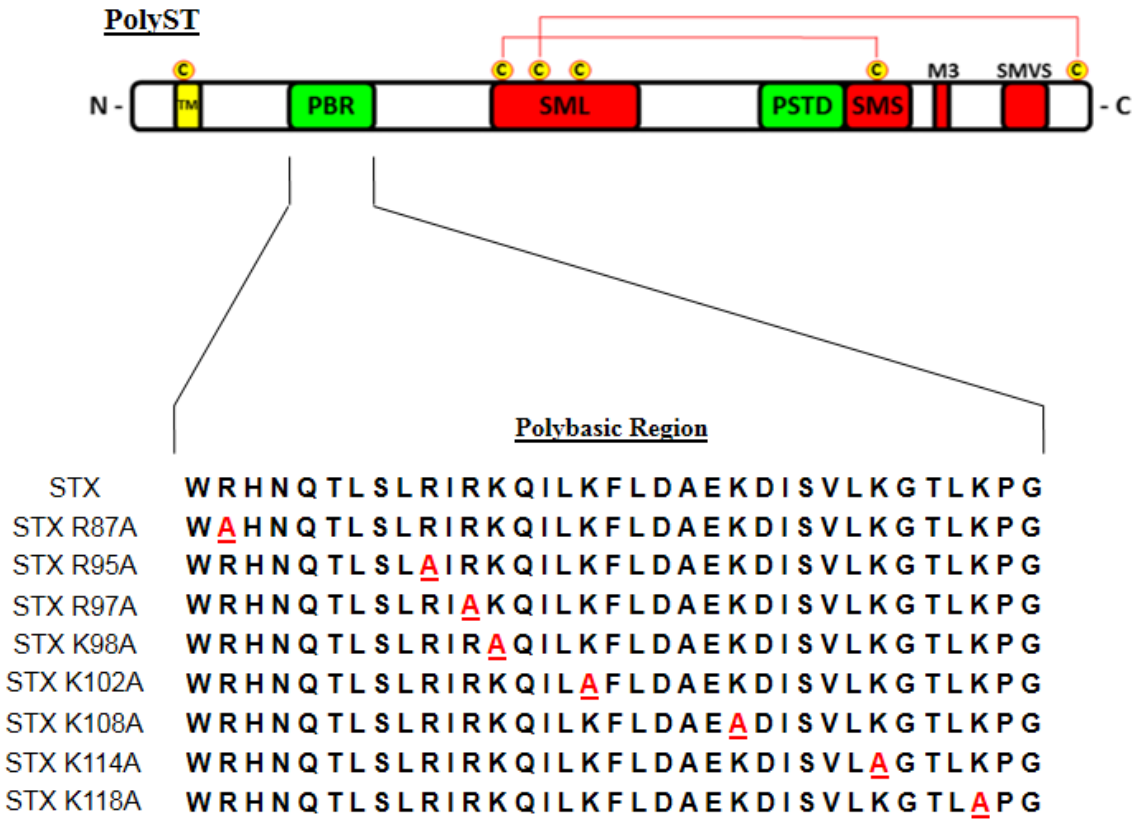

Figure 39. Creation of STX PBR alanine mutants. Each arginine and lysine residue in the STX PBR was individually mutated to alanine, leading to the creation of each of the above constructs. The location of each mutation in each STX construct is marked with an underlined red “A”.

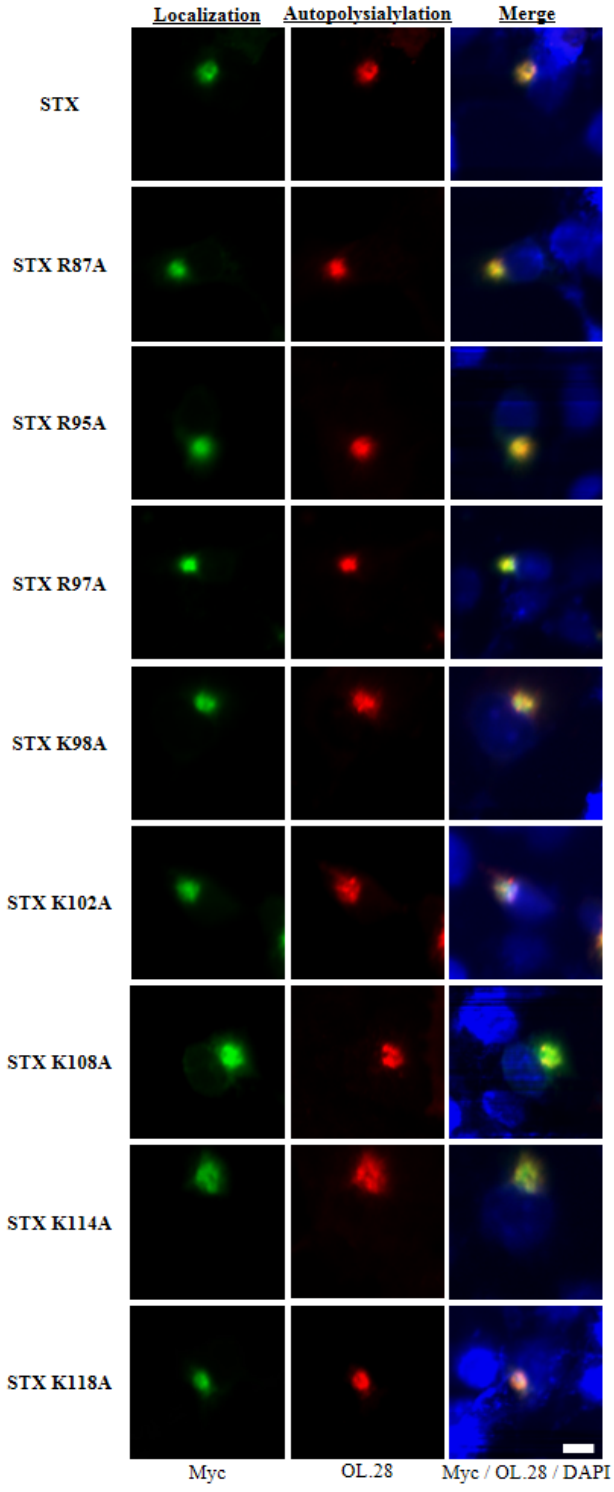

Figure 40. Similar to wild-type STX, all STX PBR mutants are predominately localized to the Golgi apparatus and are autopolysialylated. Myc-tagged STX or an STX PBR mutant were expressed in COS-1 cells. Cells were then washed, fixed with methanol, and subjected to staining with anti-Myc antibody (*green*)

V5-tagged substrates were precipitated from cell lysates with anti-V5 antibody, and the level of substrate polysialylation was assessed by immunoblotting with OL.28 anti-polySia antibody (Figures 41-42, *top panels*). Relative levels of substrate expression (Figures 41-42, *middle panels*) as well as overall enzyme expression (Figures 41-42, *bottom panels*), were evaluated by immunoblotting a small sample of cell lysate with anti-V5 and anti-Myc epitope tag antibodies, respectively, followed by quantification with NIH ImageJ.

Prior to examining the results, it is important to note that polysialylation of NRP-2 by STX and STX PBR mutants consistently produced inconclusive results. This was not the case for NCAM or SynCAM 1 polysialylation by STX. A possible rationale for why NRP-2 polysialylation by STX produced inconclusive results will be discussed later in this chapter. For now, only the results for NCAM and SynCAM 1 polysialylation will be discussed.

I found that mutation of Arg^{97}, Lys^{98}, Lys^{102}, and Lys^{108} individually to alanine in STX eliminates the polysialylation of NCAM (Figure 41, *top panel*, *lanes 5-8*). Of these residues, Arg^{97} is complementary to PST residue Arg^{82} and Lys^{108} is analogous to PST residue Arg^{93}. Therefore, the complementary STX PBR basic residues are also critical for NCAM polysialylation, but STX also demonstrates a requirement for two more basic residues for effective NCAM polysialylation. The situation is more complicated in the case of SynCAM 1 polysialylation by STX. SynCAM 1 polysialylation is reduced to different extents by a series of five STX PBR basic residues, including Lys^{98} through Lys^{118} (Figure 42, *top panel*, *lanes 6-10*), as compared to polysialylation by wild-type STX (Figure 42, *top panel*, *lane 1*). The effect on SynCAM 1 polysialylation is greatest when Lys^{108} in STX (analogous to PST Arg^{93}) is mutated to alanine (Figure 42, *top panel*, *lane 8*). Mutation of either of the two lysine residues nearest to Lys^{108} in either direction, i.e. Lys^{102} and Lys^{114}, also contribute significantly to SynCAM 1 polysialylation as polySia addition was reduced by 80% and 74% in the presence of these mutations, respectively (Figure 42, *top panel*, *lanes 7 and 9*). Mutation of Lys^{98} in STX to alanine reduced SynCAM 1 polysialylation by about 63%, which is still quite significant, whereas Lys^{118} makes a more modest contribution (Figure 42, *top panel*, *lanes 6 and 10*). Taken altogether, these results indicate that effective substrate polysialylation by STX requires larger sets of PBR basic residues than PST (Table XIV). Similar to PST substrate polysialylation, the sets of STX PBR basic residues

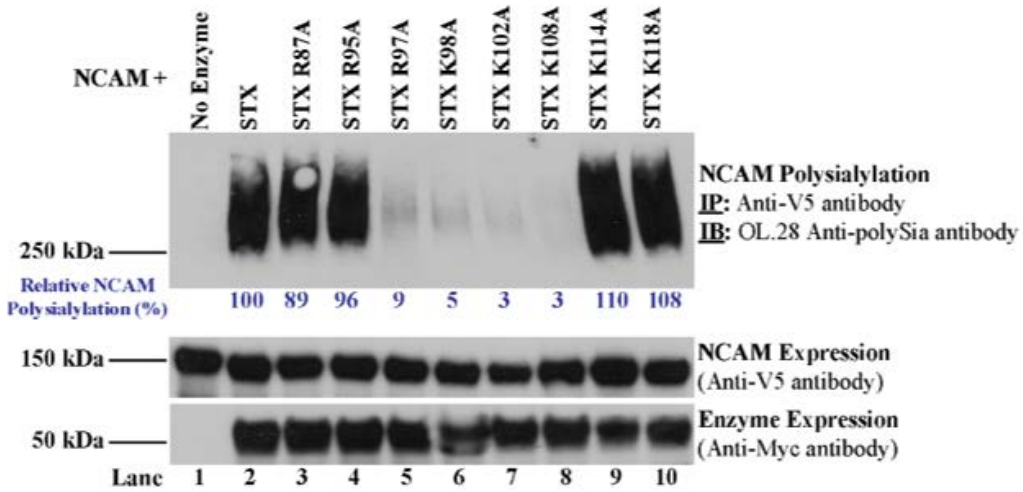

Figure 41. STX PBR residues Arg[97], Lys[98], Lys[102], and Lys[108] are required for effective polysialylation of NCAM. NCAM-V5 was co-expressed without (*lane 1*) or with Myc-tagged STX, or an STX PBR mutant (*lanes 2-10*) in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of NCAM polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

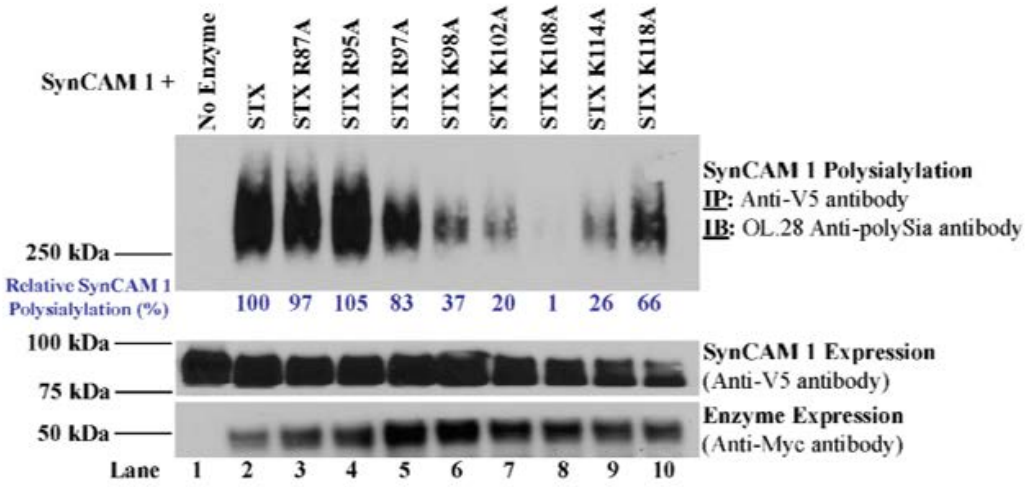

Figure 42. Effective polysialylation of SynCAM 1 by STX requires differential contributions from Lys^{98}, Lys^{102}, Lys^{108}, Lys^{114}, and Lys^{118} within the STX PBR. SynCAM 1-V5 was co-expressed without (*lane 1*) or with Myc-tagged STX, or an STX PBR mutant (*lanes 2-10*) in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates immunoprecipitated using anti-V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with OL.28 anti-polySia antibody to analyze the level of SynCAM 1 polysialylation (*top panel*). Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (*middle panel*) or anti-Myc antibody (*bottom panel*).

that were found to be critical differ by the substrate. As was the case with PST, I next determined if the identified STX residues specifically function in the recognition of NCAM and SynCAM 1.

Localization of STX H346K and STX H346K PBR alanine mutants

For competition studies, each STX PBR basic residue was mutated individually to alanine within STX H346K. Each mutant protein was then expressed in COS-1 cells and its localization evaluated by staining the cells with anti-Myc epitope antibody (Figure 43). Similar to STX and STX H346K, we find that all STX H346K PBR alanine mutants predominately localize to the Golgi apparatus with the exception of STX H346K K108A, which is not detected. This residue is analogous to Arg^{93} in PST and was found to be critical for the polysialylation of both NCAM and SynCAM 1. However, it appears that mutating this residue in STX H346K compromises protein folding and expression (Figure 43, *bottom row, second from left*). Possible rationale for a lack of expression of this protein will be discussed later, but due to the lack of expression seen, changes in the ability of STX H346K to compete with wild-type enzyme when Lys^{108} is mutated to alanine cannot be definitively interpreted.

STX PBR basic residues that are critical for NCAM and SynCAM 1 polysialylation are involved in STX-substrate recognition

As before, I expressed V5-tagged NCAM or SynCAM 1 with either Myc-tagged STX alone or with a Myc-tagged STX H346K or STX H346K PBR mutant in COS-1 cells at a ratio of 1:1:6 (NCAM:STX:STX H346K competitor) (Figures 44 and 45). The V5-tagged substrates were precipitated with anti-V5 antibody, and their polysialylation was assessed by immunoblotting with 12F8 anti-polySia antibody (Figures 44 and 45, *top panels*). Relative levels of substrate expression (Figures 44 and 45, *middle panels*), as well as overall enzyme expression (Figures 44 and 45, *bottom panels*), were evaluated and quantified as described earlier. It is important to note that overall enzyme expression refers to the combined expression of wild-type STX and an STX H346K PBR mutant.

As was seen with my competition studies utilizing PST H331K PBR mutants (Figures 32-34), PBR residues in STX that were found to be critical for substrate polysialylation also reduced the ability of STX H346K to compete with wild-type STX suggesting their role in substrate recognition. Introduction of the R97A, K98A, or K102A

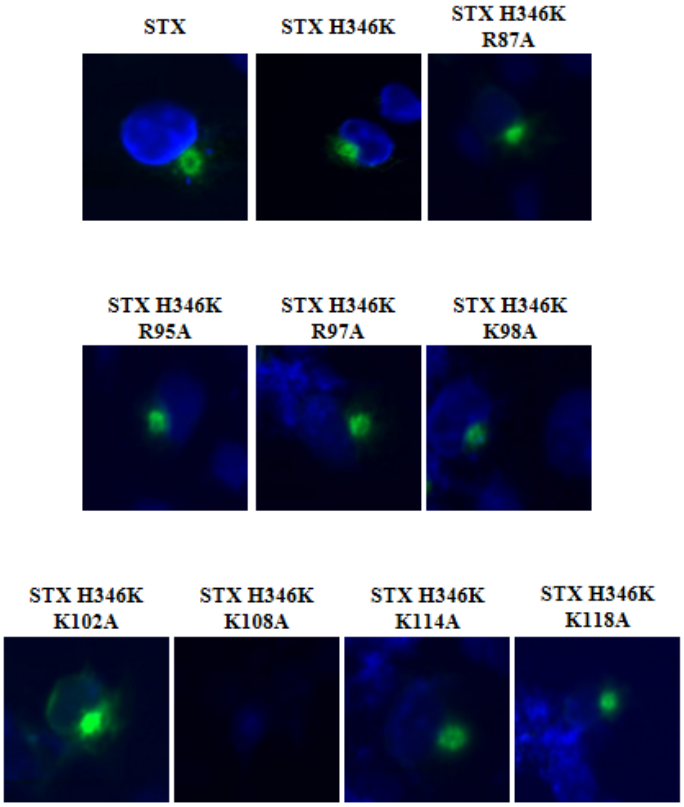

Figure 43. Similar to STX, all STX H346K PBR alanine mutants are predominately localized to the Golgi apparatus except for STX H346K K108A, which does not express. Myc-tagged STX, STX H346K, or an STX H346K PBR mutant were expressed in COS-1 cells. Cells were then washed, fixed with methanol, and subjected to staining with anti-Myc antibody (*green*) to determine protein localization. DAPI staining was utilized to indicate the location of the nucleus (*blue*). Scale bar = 10 μm.

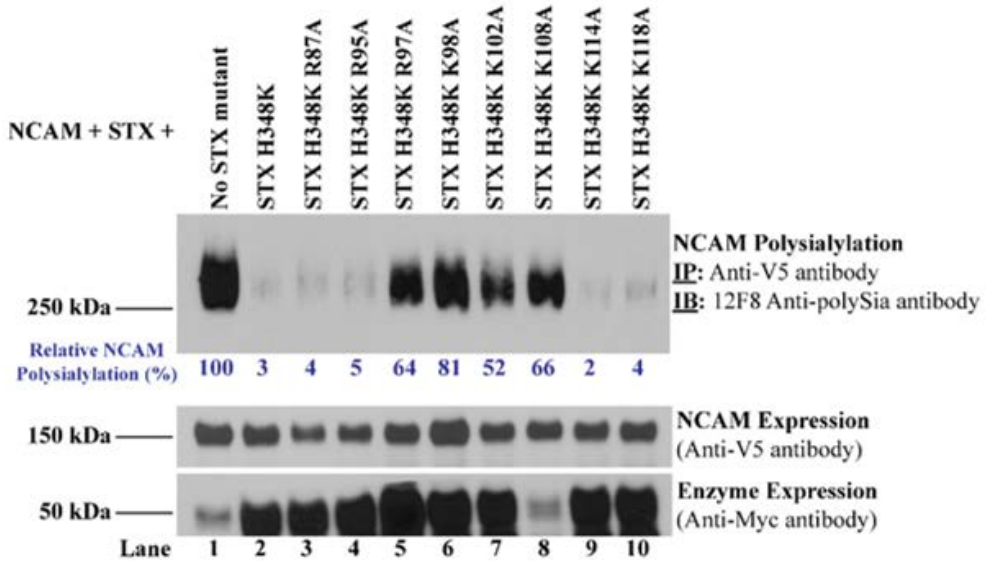

Figure 44. The ability of STX H346K to inhibit the polysialylation of NCAM by wild-type STX is compromised when Arg[97], Lys[98], and Lys[102] are replaced with alanine residues. V5-tagged NCAM and Myc-tagged STX were expressed in COS-1 cells without (*lane 1*) or with Myc-tagged STX H346K, or an STX H346K PBR alanine mutant in a 1:1:6 ratio (*lanes 2-10*). Eighteen hours post-transfection, cells were lysed and NCAM was immunoprecipitated using anti-V5 antibody. Immunoprecipitated NCAM was subjected to SDS-PAGE and immunoblotting to analyze NCAM polysialylation (*top panel*). Relative NCAM, STX and STX mutant protein expression levels were assessed by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE and immunoblotting with anti-V5 antibody (*NCAM Expression, middle panel*) or anti-Myc antibody (*Enzyme Expression, bottom panel*). Note that "Enzyme Expression" reflects the expression levels of both wild type and mutant STX proteins.

mutations into STX H346K reduced this protein's ability to compete, as indicated by a significant increase in NCAM polysialylation (Figure 44, *top panel*, *lanes 5-7*). A significant increase in the amount of NCAM polysialylation in the presence of STX H346K K108A is also seen, which would correlate with polysialylation data above, but has to be deemed inconclusive due to the lack of this protein's expression (Figure 44, *bottom panel*, *lane 8*).

Furthermore, introduction of the K98A, K102A, K114A, or K118A mutations into STX H346K negatively affected this protein's ability to compete with STX and reduce SynCAM 1 polysialylation (Figure 45, *top panel*, *lanes 6, 7, 9, and 10*). Although significant in all cases, mutation of Lys^{98} and Lys^{102} affected competition to a greater extent than Lys^{114} and Lys^{118}, which may suggest differences in resultant binding affinities in the presence of different PBR mutations. As was the case with NCAM, the loss of competition seen in the presence of the K108A mutation in STX H346K was deemed inconclusive (Figure 45, *top and bottom panels*, *lane 8*). Cumulatively, these data suggest that STX PBR residues that were found to be critical for NCAM and SynCAM 1 polysialylation are functioning specifically for the recognition of these substrates.

Discussion

In this chapter, I expanded my investigations by evaluating the criteria within STX that is critical for the recognition and polysialylation of NCAM, SynCAM 1, and NRP-2. I performed initial competition and binding studies to determine if the role of STX in substrate recognition could be similar to that of PST. From these experiments, I found that inactive STX H346K cross-competed with wild-type PST, and prevented the polysialylation of NCAM (Figure 37). As a side note, this particular result was also interesting for the fact that it also suggests that PST and STX utilize either the same or overlapping NCAM surfaces for interaction. I also found that like PST and PST H331K, STX and STX H346K were capable of binding to NCAM (Figure 38). Together, these results suggested that STX contains sequences and amino acid residues that are important for NCAM recognition and binding like PST. I subsequently proceeded with an analysis of the STX PBR basic residues and their role in the recognition and polysialylation of NCAM, SynCAM 1 and NRP-2. With the latter substrate providing inconclusive data, we decided to focus our attention on NCAM and SynCAM 1. Similar to PST, we found

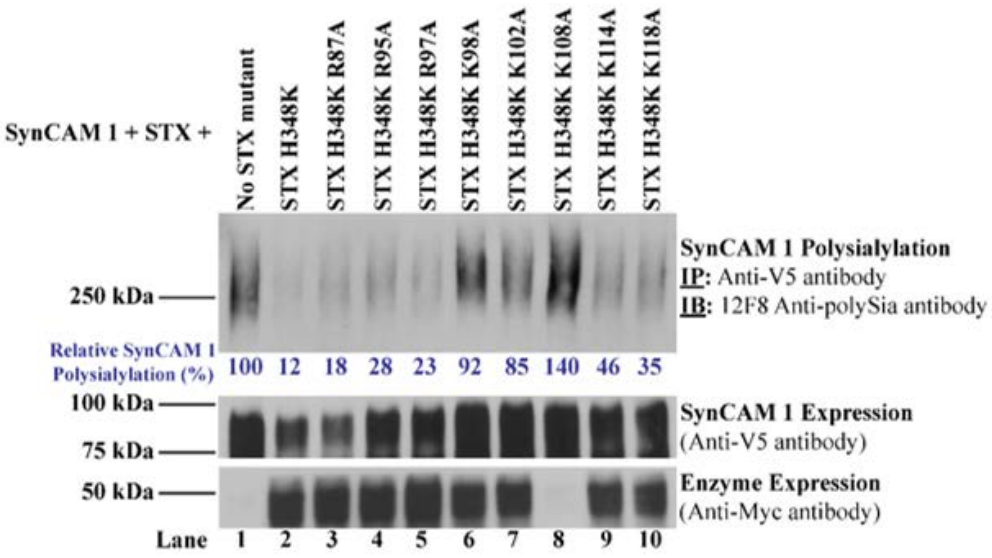

Figure 45. The ability of STX H346K to inhibit the polysialylation of SynCAM 1 by wild-type STX is compromised highly by Lys^{98}, and Lys^{102}, as well as modestly by Lys^{114} and Lys^{118} alanine mutants. V5-tagged SynCAM 1 and Myc-tagged STX were expressed in COS-1 cells without (*lane 1*) or with Myc-tagged STX H346K, or an STX H346K PBR alanine mutant in a 1:1:6 ratio (*lanes 2-10*). Eighteen hours post-transfection, cells were lysed and SynCAM 1 was immunoprecipitated using anti-V5 antibody. Immunoprecipitated SynCAM 1 was subjected to SDS-PAGE and immunoblotting to analyze SynCAM 1 polysialylation (*top panel*). Relative SynCAM 1, STX and STX mutant protein expression levels were assessed by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE and immunoblotting with anti-V5 antibody (*SynCAM 1 Expression, middle panel*) or anti-Myc antibody (*Enzyme Expression, bottom panel*). Note that "Enzyme Expression" reflects the expression levels of both wild type and mutant STX proteins.

that select STX PBR residues were important for NCAM polysialylation (Figure 41), including Arg^{97} and Lys^{108}, which are analogous to Arg^{82} and Arg^{93} in PST. Unlike PST though, two other PBR lysine residues, Lys^{98} and Lys^{102}, were critical for NCAM polysialylation by STX. The analogous PST residues for these two lysines, namely Lys^{83} and Arg^{87}, were not as significantly required for polySia addition to NCAM by PST, although there was only a slight drop in NCAM polysialylation in the presence of the K83A and R87A mutations in PST (Figure 28). The polysialylation of SynCAM 1 by STX mutants provided the most complicated data set, as five lysine residues (Lys^{98}, Lys^{102}, Lys^{108}, Lys^{114}, and Lys^{118}) were critical for effective polysialylation but to different extents (Figure 42). As such, the K108A mutation eliminated SynCAM 1 polysialylation, whereas Lys^{98}, Lys^{102}, and Lys^{114} significantly reduced polysialylation, and Lys^{118} moderately affected it. Furthermore, the STX PBR residues shown to be important for NCAM or SynCAM 1 polysialylation above, also were required for STX H346K to effectively compete with wild-type STX and reduce substrate polysialylation, suggesting that the STX PBR is also required for substrate recognition (Figures 44 and 45).

When taken together with the results from PST, we conclude that the polyST PBR is important for the polysialylation of NCAM, SynCAM 1 and NRP-2 (in the case of PST) because specific basic residues within this region function specifically for the recognition of each substrate. Also, the fact that STX requires a larger set of PBR residues than PST for recognition and polysialylation of each substrate may suggest that despite the similarities between the two polySTs, slight differences in overall biochemical mechanism may exist. Furthermore, the combination of PBR basic residues that are critical differs by the polyST-substrate combination, which correlates with the hypothesis that protein polysialylation is a highly protein-specific process. A summary of the PST and STX PBR residues that are critical for the recognition and polysialylation are listed below in Tables XV and XVI.

As was the case with a few PST PBR mutants discussed in Chapter V, there are two examples here in which the ability of a specific residue to compete does not necessarily match its ability to affect overall polysialylation (Table XVII). Here, the K102A mutation in STX nearly ablates NCAM polysialylation (97%, Figure 41, *top panel*, *lane 7*) but competition from STX H346K K102A is only diminished by 49% compared to STX H346K (Figure 44, *top panel*, *lanes 2 and 7*). Secondly, mutation of K114A in STX reduces SynCAM 1 polysialylation by 74% (Figure

TABLE XV: SUMMARY OF POLYST PBR RESIDUES REQUIRED FOR EFFECTIVE SUBSTRATE RECOGNITION AND POLYSIALYLATION ARRANGED BY SUBSTRATE

Substrate	Enzyme	PBR Basic Residues Critical for Polysialylation
NCAM	**PST**	Arg^{82} Arg^{93}
NCAM	**STX**	Arg^{97} Lys^{98} Lys^{102} Lys^{108}
SynCAM 1	**PST**	Arg^{82} Arg^{87} Lys^{99}
SynCAM 1	**STX**	Lys^{98} Lys^{102} Lys^{108} Lys^{114} Lys^{118}
NRP-2	**PST**	Arg^{82} Lys^{99}

TABLE XVI: SUMMARY OF POLYST PBR RESIDUES REQUIRED FOR EFFECTIVE SUBSTRATE RECOGNITION AND POLYSIALYLATION ARRANGED BY ENZYME

Substrate	Enzyme	PBR Basic Residues Critical for Polysialylation
NCAM	**PST**	Arg^{82} Arg^{93}
SynCAM 1	**PST**	Arg^{82} Arg^{87} Lys^{99}
NRP-2	**PST**	Arg^{82} Lys^{99}
NCAM	**STX**	Arg^{97} Lys^{98} Lys^{102} Lys^{108}
SynCAM 1	**STX**	Lys^{98} Lys^{102} Lys^{108} Lys^{114} Lys^{118}

TABLE XVII: COMPARISON OF REDUCED POLYSIALYLATION AND COMPETITION LEVELS OBSERVED FOR TWO SPECIFIC STX PBR ALANINE MUTANTS

		Substrate Polysialylation	Competition		
Substrate	**STX H346K PBR Mutant**	**Percent Polysialylation Lost**	**Percent Polysialylation w/ STX H346K**	**Percent Polysialylation w/ STX H346K PBR Mutant**	**Percent Competition Lost**
NCAM	K102A	**97**	3	52	**49**
SynCAM 1	K114A	**74**	12	46	**34**

42, *top panel*, *lane 9*), but STX H346K K114A only reduces competition by 34% compared to STX H346K (Figure 45, *top panel*, *lanes 2 and 9*). A possible rationale for this observation was discussed in detail in Chapter V. Briefly, effective polysialylation may require a completely stable substrate interaction, and therefore any change in binding affinity that may occur would not allow the STX-substrate interaction to persist long enough to allow for polysialylation. However, if binding affinities are affected only to smaller effects in the presence of a select PBR mutation, then a loss of competition may not be as significant as the corresponding loss of polysialylation observed with that mutation.

Here, I found that STX H346K K108A did not demonstrate any protein expression (Figure 43). This was quite unfortunate, as the corresponding residue in PST (Arg^{93}) was critical for the recognition of NCAM, and the inability to detect the double mutant did not allow me to analyze the role of Lys^{108} in substrate recognition. It is likely that the STX H346K K018A protein is expressed but quickly degraded possibly due to gross misfolding. What is more interesting is that the same mutation in wild-type STX did not significantly affect protein folding, as STX K108A was localized to the Golgi and autopolysialylated (Figure 40). A possibly explanation could be that STX residues Lys^{108} and His^{346} may be involved in a long-range interaction that is highly critical for the overall

stability of STX. Whether this possible interaction is via a series of hydrogen bonds or salt bridges cannot be determined without a crystal structure of STX, but a long-range interaction could be envisioned. Angata *et al.* [307] found that two disulfide bridges within the polySTs, which are also diagrammed in Figure 7, are necessary for autopolysialylation and substrate polysialylation. Mutating the cysteine residues to alanines, or truncating the C-terminus of PST closer than Cys^{356}, abolished all activity of PST. These disulfide bridges can be theorized to effectively "loop" the polyST's C-terminal regions back over the middle of the protein, such that the catalytic sialylmotifs are in proper alignment in order to synthesize polySia. With this in mind, it is possible that His^{346} and Lys^{108} could come close enough to form a portion of a critical interacting set of residues. Long-range interactions between sets of residues have previously been shown to be critical for overall protein stability. For example, this was shown for hen's-egg white lysozyme in which point mutations in residues mediating a long-range interaction significantly destabilizes the entire enzyme [335]. In this case, it is plausible that mutation of His^{346} or Lys^{108} alone may still allow for the protein to remain in a stable configuration, but mutation of both in tandem disrupts a critical long-range interaction. This may also be the case seen in Chapter V with PST H331K K83A, which expressed but was predominately expressed in the endoplasmic reticulum.

As mentioned above, I had difficulty evaluating NRP-2 polysialylation by STX. The most straightforward explanation stems from studies that indicate that NRP-2 polysialylation is found in dendritic cells with concurrent upregulation of the gene that codes for the ST8Sia IV/PST protein [132] and not that of ST8Sia II/STX, suggesting that under physiological conditions, NRP-2 is exclusively polysialylated by PST. This would further suggest that the inconsistent polysialylation that we observed may be an artifact of high levels of STX and NRP-2 expression. A second possibility comes from recent unpublished results from our laboratory. The laboratory found that an acidic surface patch in the NCAM FN1 domain is critical for polyST polysialylation and binding [297, 299]. In a search for residues within NRP-2 that may potentially mediate polyST recognition, we have found several patches of acidic residues located on the surface of the MAM domain. These patches have not been fully characterized yet but one possibility is that when STX and STX PBR mutants are highly over-expressed with NRP-2 in our COS-1 expression system, the STX proteins may be capable of interacting in different ways with multiple acidic residues where only

certain interactions will lead to NRP-2 polysialylation. Consequently, differences in level of enzyme and substrate expression may alter efficiency of polysialylation and the impact of single point mutations in the STX PBR.

In conclusion, the findings presented here represent a thorough analysis of the role and relevance of all of the polyST PBR basic residues for the polysialylation of multiple substrates including NCAM, SynCAM 1 and NRP-2. These results undoubtedly indicate that the polyST PBR, which is not part of the catalytic sialylmotifs, is necessary for substrate recognition, and for this reason, the PBR is necessary for overall substrate polysialylation. It is important to note that the analyses presented in this thesis, although critical to understanding the biochemical mechanism of substrate polysialylation, represent the identification of only one region of the polySTs that is necessary for recognition. With our laboratory's recent finding that residues in the NCAM Ig5 domain may also function as a secondary recognition site, more work is required to examine other regions between the catalytic sialylmotifs to determine if they contain residues that complement those in the PBR and function in mediating a potential secondary substrate recognition site.

CHAPTER VII

CONCLUSIONS AND FINAL REMARKS

The human polySTs, PST and STX, catalyze the synthesis of polySia on the termini of N- or O-linked glycans found on only seven glycoproteins, including NCAM, their major substrate, SynCAM 1, NRP-2, and the polySTs themselves, to name a few. The role of polySia on NCAM has been extensively studied, and it has been found that polySia plays roles in modulating NCAM's cell-cell adhesive abilities and cell signaling properties. Over the past several decades, the absolute necessity of polysialylated NCAM in all stages of life has been well documented. PolyST knockout mice examined by Weinhold *et al.* [198] demonstrated that polysialylated NCAM is necessary for the proper pre-natal development of the mammalian nervous system and survival after birth. Furthermore, in multiple studies that I detailed in Chapter I, polySia has been shown to be critical for memory formation, smell, and neuronal repair in the adult mammal. More recent studies have also described potential roles for polySia in SynCAM 1's ability to mediate adhesion at the neuronal-glial synapse [289], while its modification of NRP-2 is suggested to promote inhibition of dendritic cell-induced T cell activation and proliferation or enhance dendritic cell migration to lymph nodes [293,294].

Of all the findings mentioned above in which polySia has played necessary and beneficial roles throughout mammalian life, studies demonstrating potentially detrimental roles of polySia in human disease and cancer were even more intriguing. Levels of polySia have been found to be unnaturally altered in several disease states, including schizophrenia [252-256], Alzheimer's disease [261-264], epilepsy [267, 268], and Huntington's disease [272-274] (see Chapter I). Furthermore, polySia has been found to be re-expressed in the late stages of a variety of human tumors both within and outside the nervous system, where invasion and metastasis is common [reviewed in 275]. These findings confirm that polysialylation is a very critical biological process that needs to be fully understood, and raises the question as to whether we could manipulate this process to promote proper development, function, or inhibit the process as a therapeutic in various human diseases such as cancer. However, at the beginning of this thesis work, the precise biochemical mechanism by which substrate polysialylation occurs was quite unclear.

When this thesis work began, it was known from previous NCAM deletion, structural, and mutational analyses from our laboratory and others (see Chapter I) that the NCAM FN1 domain and residues within it are necessary for the effective polysialylation of N-glycans in the neighboring Ig5 domain [311-313, 317, 318]. Furthermore, it was found that PST binds to NCAM and this interaction is eliminated or significantly reduced when the NCAM FN1 domain is either not present or has specific surface acidic residues mutated to alanines, respectively [314], signifying that the FN1 domain mediates an important contact with PST. The observations encouraged our laboratory to examine PST for the presence of residues that would potentially form the complimentary second half of the proposed interaction. It was found that two basic residues in the PST PBR, Arg^{82} and Arg^{93}, were critical for the effective polysialylation of NCAM despite not being within the catalytic sialylmotifs [305]. From these NCAM and PST studies originated the proposal that has served as the overarching theme of this thesis work—that substrate polysialylation is highly substrate specific and requires an initial recognition step that positions the polyST and substrate accordingly for effective polysialylation to occur.

At the start of this thesis work in 2008, there was not much information outside of our laboratory's data concerning understanding the sequences within the polySTs that are necessary for mediating this recognition step. It is for this reason that in my thesis work I sought to specifically identify and analyze polyST sequences that are critical for substrate recognition and may be complimentary to the acidic sequences originally found in the NCAM FN1 domain. Since this work began, a secondary interaction involving residues in the NCAM Ig5 domain has been identified [316]. However, this interaction is clearly secondary and not well-characterized. My work has focused on the interaction of the polySTs with the NCAM FN1 domain acidic patch residues that is absolutely necessary for polysialylation to occur.

Focusing on the region prior to the large sialylmotif in the polySTs, I created a set of catalytically-inactive full-length and truncated PST proteins and utilized SW2 cell competition studies and co-immunoprecipitation assays to specifically identify a region within PST that is critical for NCAM recognition and binding. Once a region was identified, I created specific point mutations in PST PBR basic residues and utilized the resultant mutants in an in cell competition assay and in polysialylation studies to more thoroughly examine this PST region for specific

amino acids that mediate the recognition of NCAM. In keeping with very recent studies in the field, I expanded these analyses to include two other polysialylated substrates—SynCAM 1 and NRP-2. PST only represents half the story, as several human diseases and cancers show a preferential expression of STX. Therefore, I also analyzed the other polyST, STX, for residues critical for NCAM and SynCAM 1 recognition and polysialylation. Below, I will outline and discuss the major findings that have come from this work.

Identification of a region in PST that is critical for NCAM recognition

This thesis work began with the goal of identifying a specific region within PST that was critical for NCAM recognition (Chapter III). To accomplish this task, I created a series of catalytically-inactive PST mutant proteins consisting of one full-length PST protein containing one point mutation in His^{331} (PST H331K) and a series of truncated PST mutants consisting of the first 140, 127, 105, 71, and 62 residues of PST, all of which do not contain any of the catalytic sialylmotifs (PST140, PST127, PST105, PST71, and PST62), and a catalytically active PST and truncated catalytically inactive soluble PST (sPST and sPST140). Since PST105 did not express, this protein was not utilized in any further experiments. To specifically determine if these PST proteins contained sequences that were critical for NCAM recognition, we expressed wild-type PST and each PST mutant protein individually in SW2 small cell lung carcinoma cells which contain endogenous PST and polysialylated NCAM. My rationale for this assay was that if a particular catalytically-inactive PST protein still contained sequences critical for NCAM recognition, then when expressed in SW2 cells, this PST protein would interact with NCAM, effectively preventing the interaction of endogenous PST with NCAM and resulting in a reduction in surface NCAM polysialylation. A reduction in SW2 surface polysialylation was the readout used to identify the presence of competition.

I found that PST and sPST increased SW2 cell surface polysialylation, as would be expected for expression of excess catalytically-active proteins. Expression of PST H331K significantly reduced SW2 cell surface polysialylation, as did PST140 and sPST140, suggesting that these three proteins are competing with endogenous PST. Since PST140 and sPST140 reduced SW2 NCAM polysialylation similar to the reduction seen with PST H331K, I predicted that the PST region between the end of the transmembrane region (residue 21) and the start of the large sialylmotif (residue 141) is critical for NCAM recognition. Furthermore, SW2 cell NCAM polysialylation

was also significantly reduced in the presence of PST127 expression, but not in the presence of PST71 or PST62 expression, suggesting that the latter two proteins no longer contain sequences critical for NCAM recognition. Overall, these results suggest that residues 71-127 of PST compose a region that contains criteria necessary for the recognition of NCAM.

The premise of these competition studies is that catalytically-inactive PST mutant proteins compete via interaction with SW2 cell NCAM, thus preventing interaction between NCAM and wild-type endogenous PST. Could there be another explanation for the observed competition and reduction in cell surface NCAM polysialylation? One alternative possibility is based on the fact that some glycosyltransferases, including the mammalian medial Golgi enzymes N-acetylglucosaminyltransferase I and II (GnTI and GnTII), β-1, 4-galactosyltransferase, select fucosyltransferases, and sialyltransferases ST3Gal I and ST6Gal I, as well as the rat liver α2, 6-sialyltransferases, have been shown to form dimers [336-340]. Although there is no evidence that the human polySTs form dimers, the fact that the polySTs are autopolysialylated suggests that the formation of a dimer is possible. In terms of our competition studies, polyST dimerization is important to consider because in the event that formation of a polyST homodimer is required for catalytic activity or NCAM recognition, then dimerization of an overexpressed catalytically-inactive PST mutant protein with endogenous PST could mimic competition by preventing endogenous PST from reaching NCAM or leading to its inactivation. This could easily sequester all of the SW2 cell's PST since the catalytically-inactive PST mutants are expressed in high excess.

Although more work is required to determine if the polySTs are capable of forming dimers under physiological conditions, from several previous studies as well as observations from the SW2 cell competition studies presented in this thesis work, I believe that the results obtained from these competition experiments is a result of an interaction between NCAM and a catalytically-inactive PST mutant. In a few previous studies, it has been shown that sialyltransferases form a dimer through their transmembrane region, but catalytically active soluble forms of these enzymes retain catalytic activity [336, 339], which would imply that dimerization is not required for catalytic function of the polySTs assuming that if a dimer forms in the polySTs that it involves transmembrane region sequences. In my competition studies, sPST was still capable of raising SW2 surface NCAM polysialylation

similar to excess wild-type PST, correlating with this previous result. Furthermore, sPST140 is able to compete with endogenous PST and significantly reduce SW2 cell NCAM polysialylation similarly to PST H331K and PST140, suggesting that a potential enzyme dimerization event is not critical for NCAM recognition. Finally, an argument against enzyme dimerization occurring to allow for autopolysialylation is that that our catalytically-inactive PST mutant proteins containing N-glycans utilized for autopolysialylation (PST H331K, PST140, sPST140, and PST127) are not polysialylated by SW2 cell PST. We also attempted in the laboratory to find an interaction between Myc-tagged and V5-tagged PST proteins with co-immunoprecipitation experiments, which did not demonstrate binding (data not shown). For these reasons, it is likely that the competitive effect observed by catalytically-inactive PST mutant proteins in SW2 cells is more likely due to a direct interaction of each PST mutant with NCAM and not endogenous PST.

To determine if the ability of specific PST sequences to successfully compete with endogenous PST and reduce NCAM polysialylation was due to the ability of a particular sequence to bind to NCAM, I utilized my PST mutants in co-immunoprecipitation studies. I found that comparable to wild-type enzyme, PST H331K, PST140, and PST127 were able to bind to NCAM. Soluble PST and sPST140 were also able to bind to NCAM, suggesting that the transmembrane region and cytosolic tail were not significant for PST-NCAM interaction. Furthermore, PST71 and PST62 did not effectively bind to NCAM. Cumulatively, these data suggest that the PST region between residues 71 and 127 contains specific residues that are critical for PST binding to NCAM. These data correlate directly with our SW2 studies above, where the ability to compete with endogenous PST remains in all PST mutant proteins containing residues 71-127, but is lost in mutants containing only the first 71 or 62 residues of PST (PST71 and PST62).

The ability of a particular PST sequence to recognize and bind to NCAM is most likely due to a direct interaction between PST and NCAM, and does not involve an intervening molecule. Two current pieces of data support this idea. First, utilizing purified soluble STX (sSTX), and a GFP-tagged soluble PST (GFP-PST) obtained from Dr. Kelley Moremen (University of Georgia-Athens), I was able to demonstrate in an *in vitro* assay containing V5-tagged soluble NCAM (sNCAM) purified from COS-1 cells, a purified enzyme, and CMP-sialic acid, that both

enzymes we able to effectively polysialylate sNCAM, and that the polysialylation occurred on N-linked glycans as determined by Endo N treatment (data not shown). Secondly, recent isothermal titration calorimetry experiments from the laboratory indicated that a peptide consisting of the PST PBR region (residues 71-105) was capable of binding to the NCAM Ig5-FN1 unit, and that this binding was most likely a result of electrostatic interactions (data not shown). This recent data could suggest the interaction of basic residues within the PST PBR with acidic residues on the Ig5-FN1 substrate.

In sum, my earliest studies indicated that residues 71-127 of PST, a region outside the catalytic sialylmotifs, contains specific sequences that are critical for the effective recognition of and interaction with the NCAM substrate. Next, I identified specific residues within this region that specifically mediate substrate recognition.

Analysis of the role of two PST polybasic region residues, Arg^{82} and Arg^{93}, in NCAM recognition

Since the PST PBR was previously shown to be critical for NCAM polysialylation in general [305], and since the PBR falls completely within the PST region above, I decided to focus my attention on the PST PBR in my search for residues that are critical for NCAM recognition. With the knowledge that the NCAM FN1 domain contains surface acidic residues that are critical for NCAM polysialylation and binding by PST [312, 314], I initiated my search in Chapter IV by focusing on the basic residues within the PST PBR. The two initial residues evaluated were Arg^{82} and Arg^{93}, which were shown to be required for effective NCAM polysialylation [305].

To determine if Arg^{82} and Arg^{93} functioned specifically for NCAM recognition, I mutated each residue to alanine either singly or doubly within PST H331K and utilized the SW2 cell competition assay as previously described (PST H331K R82A, PST H331K R93A, PST H331K R82A/R93A). Since PST H331K competes with endogenous SW2 cell NCAM and reduces NCAM polysialylation, to determine if a particular residue is specifically important for recognition, I looked for a reduction in the ability of PST H331K to compete in the presence of these mutations, as determined by the level of cell surface polysialylation.

I found that the presence of either mutation singly or doubly reduced the ability of PST H331K to compete with SW2 cell PST and reduce NCAM polysialylation. However, the difference in competition loss between a single

mutant (PST H331K R82A or PST H331K R93A) and the double mutant (PST H331K R82A/R93A) was difficult to discern qualitatively using a confocal microscope. Since flow cytometry methods were unsuccessful using polySia antibodies, I utilized a triple expression system in COS-1 cells as a modified competition assay. In this assay, where a PST H331K competitor is expressed in excess of NCAM and wild-type PST, NCAM polysialylation could be quantified followed immunoprecipitation and immunoblotting with an antibody against polySia. Here, I found that mutation of Arg^{82} or Arg^{93} to alanine in PST H331K, significantly reduced the ability of PST H331K to compete with wild-type PST, as marked by a modest increase in NCAM polysialylation. However, when both arginine residues were mutated to alanine in tandem, the competitive effect of PST H331K was eliminated, indicating that the reason why Arg^{82} and Arg^{93} in the PST PBR are required for NCAM polysialylation is that they are both required for effective recognition of NCAM.

The finding that PST PBR residues Arg^{82} and Arg^{93} were critical for NCAM recognition was a key finding and a good start. Therefore, I decided to next expand my studies by including (1) an analysis of all of the PST PBR residues for NCAM recognition, and (2) an analysis of the significance of each PST PBR basic residue for the effective recognition and polysialylation of SynCAM 1 and NRP-2. I included SynCAM 1 and NRP-2 in these studies because polySia was recently found to modulate key functions of these proteins [289, 293, 294], and the mechanism by which they are polysialylated was unknown and not studied previously.

Determination of the role of PST polybasic region basic residues in recognition and polysialylation of NCAM, SynCAM 1, and NRP-2

In the second portion of Chapter IV, I found that PST residue Arg^{82} was universally important for the polysialylation of NCAM, SynCAM 1, and NRP-2, whereas Arg^{93} was only required for NCAM polysialylation, raising the question as to whether different combinations of residues are critical for polysialylation of distinct substrates. To definitively answer this question, in Chapter V I sought to determine the significance of each PST PBR basic residue for the recognition and polysialylation of NCAM, SynCAM 1, and NRP-2. For this purpose, I created a series of alanine mutants in which each PST PBR basic residue was mutated singly to alanine in PST for polysialylation studies and in PST H331K for competition studies. I found that as in the past, Arg^{82} and Arg^{93} were

the two PST residues that were most critical for NCAM polysialylation. However, I also found that SynCAM 1 polysialylation required PST residues Arg^{82}, Arg^{87}, and Lys^{99}, whereas NRP-2 polysialylation required Arg^{82} and Lys^{99}. These results suggest that PST Arg^{82} plays a more general role in substrate polysialylation, whereas Arg^{87} and Arg^{93} have more specific roles for SynCAM 1 and NCAM polysialylation, respectively, and Lys^{99} is required for polysialylation of both SynCAM 1 and NRP-2.

Utilizing competition analyses as in Chapter V, I next sought to determine if these residues that are required for polysialylation of each substrate are functioning specifically in their recognition. I found that the residues identified above for the polysialylation of each substrate were the same residues involved in recognition. For example, mutation of Arg^{82} or Arg^{93} to alanine in PST H331K were the only residues to definitively reduce the ability of this PST mutant to compete with wild-type PST, leading to an increase in NCAM polysialylation as compared to PST H331K without any PBR mutations. For SynCAM 1, mutation of PST residues Arg^{82}, Arg^{87}, or Lys^{99} reduced PST H331K's ability to compete, whereas only Arg^{82} and Lys^{99} reduced PST H331K's ability to compete in the presence of NRP-2. As discussed previously in detail, PST H331K K83A was predominately localized to the endoplasmic reticulum, indicating a potential gross misfolding and hence competition results obtained with this protein could not be interpreted.

In sum, these studies indicate two major interpretations concerning the PST PBR. First, the PST PBR is important for polysialylation of NCAM, SynCAM 1, and NRP-2 because residues within it function specifically for substrate recognition. Secondly, the precise criteria within PST that are required for effective polysialylation and recognition of NCAM, SynCAM 1, and NRP-2 is unique to each substrate, as they utilized different combinations of PST PBR basic residues. The take home message from these studies is that overlapping sets of basic residues, and hence surfaces, within the PST PBR are utilized for the recognition of NCAM, SynCAM 1, and NRP-2, correlating with the notion that protein polysialylation is a highly protein-specific biochemical process.

Identification of specific amino acids in the STX polybasic region that are critical for NCAM and SynCAM 1 recognition and polysialylation and how these residues compare to those identified in PST

In Chapter VI, I sought to determine if the results found in PST could be recapitulated in the other human polysialylatransferase, STX. I determined that STX contains sequences that are required for NCAM recognition, as I showed that STX H346K (analogous to PST H331K) was able to compete with wild-type PST and effectively ablate NCAM polysialylation. Furthermore, STX and STX H346K were both able to effectively bind to NCAM. These two results indicated that similar to the case of PST, STX contains sequences that mediate the recognition of and interaction with the NCAM substrate. From these results, I determined that the mechanism of substrate polysialylation by STX may be similar to that of PST.

To test this possibility, I created a series of STX and STX H346K point mutants in which each PBR basic residue was mutated individually to alanine. I found that effective polysialylation of NCAM by STX required PBR residues Arg^{97}, Lys^{98}, Lys^{102}, and Lys^{108}. The case was more complicated with SynCAM 1, as Lys^{98}, Lys^{102}, Lys^{108}, Lys^{114}, and Lys^{118} were all required for effective polysialylation, albeit they were each required to different extents. As discussed in detail in Chapter VI, the polysialylation of NRP-2 by STX PBR mutants produced inconclusive results, which were not further analyzed. In addition, competition analyses indicated that similar to PST, the residues identified as being critical for either NCAM or SynCAM 1 polysialylation by STX, were also required for the recognition of each respective substrate. As discussed in depth in Chapter VI, STX H346K K108A did not demonstrate any expression, suggesting that it has grossly misfolded and was most likely degraded.

In sum, I have determined that the STX PBR is important for the polysialylation of NCAM and SynCAM 1 because it functions specifically in substrate recognition. Furthermore, different sets of basic residues within the STX PBR are required for the recognition of different substrates. These two findings correlate with those found for the PST PBR in Chapters IV and V, suggesting that the biochemical mechanism of protein polysialylation is similar between the two polySTs. However, there were also a noticeable differences in requirements for PST and STX PBR residues in substrate polyisialylation. For STX, larger sets of basic residues in the STX PBR were required for substrate recognition and polysialylation, than were required for PST. As an example, effective NCAM recognition

and polysialylation by PST required two residues, Arg^{82} and Arg^{93}. The same process requires four STX PBR residues, two of which are analogous to the arginine residues found in PST (Arg^{97} analogous to Arg^{82} in PST; Lys^{108} analogous to Arg^{93} in PST) and two that are specific to STX.

Summary of major findings

In this thesis work, I sought to elucidate the biochemical mechanism of substrate polysialylation by the human polySTs by identifying sequences and residues in the enzymes that are critical for substrate polysialylation. The major conclusions of my studies are summarized here.

First, the N-terminal Golgi lumenal region prior to the large sialylmotif of the polySTs contains sequences that are critical for substrate recognition. This specifically includes residues 71-127 of PST, and potentially the entire complimentary region in STX (residues 86-142).

Secondly, the polyST PBR, which is located completely within residues 71-127 of PST and 86-142 of STX, contains specific basic residues that are critical for NCAM, SynCAM 1, and NRP-2 recognition and polysialylation.

Finally, different sets of overlapping polyST PBR basic residues are utilized as unique recognition surfaces that are specific for each polyST-substrate recognition complex. This conclusion is based on the fact that in no polyST-substrate combination tested did the same pattern of polyST PBR basic residues affect substrate recognition and polysialylation. This result also further exemplifies the high level of protein specificity that is believed to be a key component to the overall mechanism of substrate recognition and polysialylation by the polySTs.

In all, I have identified the polyST PBR, and specific criteria within, as being critical for substrate recognition. Although these findings are critical and represent a step forward toward fully understanding the biochemical mechanism of substrate polysialylation, the PBR is only a small section of the polyST and future studies will need to examine the remainder of the polyST sequences for other areas that are critical for substrate recognition. As for the precise mechanism by which the polyST PBR interacts with NCAM, SynCAM 1, and NRP-2, I now present a plausible hypothesis based on structure studies and structural modeling concerning where the polyST PBR may interact with each substrate.

Defining the specific role of polyST PBR basic residues in the process of substrate recognition

What specific sites on the substrates are the PST and STX PBR basic residues recognizing? At the current time, this question cannot definitively be answered since there is no concrete structural information regarding the three-dimensional structure of the polySTs, nor the potential binding interfaces with their substrates. However, based on previous studies and some current ongoing work in the laboratory, I can predict the mechanism by which the polyST-substrate interaction is occurring.

Interaction of the polySTs with NCAM

Previous studies from our laboratory and others demonstrated the significance of the NCAM FN1 domain in polysialylation and recognition by the polySTs, as deletion of the NCAM FN1 domain eliminates NCAM Ig5 domain polysialylation and polyST binding [311, 314]. From the crystal structure of the NCAM FN1 domain solved previously by our laboratory in collaboration with Arnon Lavie and Nikolina Sekulic [312], we identified a surface acidic patch on this domain. Mutational analysis demonstrated that these specific negatively-charged residues within FN1 are critical for NCAM polysialylation at Ig5 and for polyST binding [314]. In total these results suggest that acidic residues in the NCAM FN1 are critical for NCAM recognition by the polySTs. From the results obtained in this thesis work, it is plausible that the polyST PBR contains the complimentary sequences that will interact with the previously identified NCAM FN1 acidic patch residues. The residues identified in the PST and STX PBR regions are positively charged, which can form electrostatic interactions with the NCAM FN1 domain. Recent ITC results from our laboratory seem to suggest that this is a possibility, as a PST PBR peptide (residues 71-105) was found to bind to NCAM Ig5-FN1 with an enthalpy indicative of an electrostatic interaction (data not shown).

More recent studies from our laboratory suggest that the polySTs also must interact with select residues in the NCAM Ig5 domain [316]. In an effort to determine why the olfactory cell adhesion molecule is not polysialylated, despite possessing a FN1 domain that is able to partially substitute for NCAM FN1 to allow polyST recognition, our laboratory first showed that the OCAM Ig5 domain was not permissive for polysialylation despite having appropriately placed N-glycans [316]. Next, we discovered that two basic residues nearby the two

asparagines that are analogous to the residues carrying NCAM polysialylation glycans are prohibitive for OCAM polysialylation. Specifically, replacement of Ser^{448} and Asn^{476} in NCAM Ig5 with the lysine and arginine residues found in OCAM eliminated NCAM polysialylation and reduced PST binding, suggesting a role for these residues as parts of a larger polyST recognition surface. However, we believe that this polyST-Ig5 interaction is secondary to a polyST-NCAM FN1 interaction, as NCAM Ig5 is only polysialylated in the presence of the FN1 domain, and not when it stands alone [311].

The study described above also highlighted the importance of the FN1 acidic patch in NCAM recognition and polysialylation by showing that the creation of the full acidic patch in OCAM FN1, by the insertion of just two acidic residues, substantially increased the polysialylation of an OCAM chimera containing the NCAM Ig5 domain [316]. I believe that the polyST PBR is recognizing this major interaction site at the NCAM FN1 domain. The polyST PBR is located in the N-terminal Golgi lumenal region of the polyST, prior to any of the catalytic sialylmotifs. The "catalytic region" of the polySTs encompassing the region between the start of the large sialylmotif and the C-terminus of PST or STX, is quite large, spanning 218 residues in PST and 219 residues in STX. Two disulfide bridges between cysteine residues are critical for overall polyST protein stability and activity [307]. Although we don't have a crystal structure of a polyST, one can envision that these disulfide bridges likely cause the C-terminal sequences of the polyST to effectively "loop" back over the sequences near the beginning of the catalytic region, such that the sialylmotifs are positioned properly relative to each other to form an active site and to allow for effective substrate polysialylation. Envisioning this, it is plausible that as the polyST PBR interacts with the NCAM FN1 domain, the three-dimensional structure formed by these disulfide bridges and containing the constellation of sialylmotifs would be located in the proximity of the NCAM Ig5 domain where polysialylation occurs. From this, I predict that the polyST PBR interacts with sequences in the NCAM FN1 domain, whereas later polyST sequences between the sialylmotifs are responsible for making secondary contacts with sequences in NCAM Ig5.

In sum, it is likely that the polyST PBR and specific basic residues within first interact with acidic surface residues in the NCAM FN1 domain (Figure 46, "*Predicted NCAM-polyST interaction*"). This interaction then

stabilizes the polyST next to NCAM, which then allows for the polyST to interact with sequences in the NCAM Ig5 so as to properly align its active site with Ig5 N-glycans, allowing for effective NCAM polysialylation to occur.

Interaction of the polySTs with SynCAM 1

SynCAM 1 is polysialylated on a single N-glycan within the Ig1 domain [289, 341]. Rollenhagen *et al.* [342] utilized a series of SynCAM 1 constructs to determine the minimal structural domains that are required for the effective polysialylation found at Ig1. Following purification of three SynCAM 1 constructs consisting of (1) all three Ig domains, (2) Ig1 and Ig2, or (3) Ig1 alone, each was subjected to polyST polysialylation *in vitro*. It was found that a SynCAM 1 protein consisting of all three Ig domains or just Ig1 and Ig2, were effectively polysialylated. However, Ig1 alone was not. From these studies, it was concluded that the SynCAM 1 Ig1-Ig2 tandem is the minimal structural unit required for SynCAM 1 polysialylation.

This result was compelling because it suggests that the neighboring Ig2 domain in SynCAM 1, which is not the location of polysialylation, is somehow required for polysialylation. This situation is analogous to the NCAM Ig5-FN1 tandem, where the neighboring FN1 is required for polysialylation at NCAM Ig5. Could the SynCAM 1 Ig2 domain be analogous to NCAM Fn1 and function as a docking site for the polySTs? To shed more light on this possibility, our laboratory modeled the SynCAM 1 extracellular domain (Ig1-Ig2-Ig3) based on the NCAM Ig1-Ig2-Ig3 structure (PDB ID: 1QZ1) in an effort to determine if there were potential acidic surfaces on Ig2 capable of interacting with the polySTs (data not shown). We found that the SynCAM 1 Ig2 domain contained two sets of acidic surfaces that could potentially be part of a polyST interaction. First, Glu^{163}, Glu^{164}, and Glu^{166} in the SynCAM 1 Ig2 model were identified as a potential acidic recognition surface for the polySTs. Secondly, a small helix in SynCAM 1 Ig2 consisting of three acidic residues, Glu^{194}, Glu^{196}, and Glu^{197}, may play a role analogous to the unique a-helix in NCAM FN1 that is believed to help to properly position the polyST. More recent mutational analysis in our laboratory indicate that several of these acidic residues when mutated to alanines reduces SynCAM 1 polysialylation by PST (unpublished results), suggesting that an acidic surface in SynCAM 1 Ig2 may function for polyST recognition and docking.

From these studies detailed above, I hypothesize that the polyST PBR may recognize and interact with specific acidic residues within the SynCAM 1 Ig2 domain, thus stabilizing the polyST next to SynCAM 1 in a way similar to NCAM FN1 and allowing for polysialylation to occur at the neighboring Ig1 domain (Figure 46, *"Predicted SynCAM 1-polyST interaction"*). Any secondary interaction between the polyST and the SynCAM 1 Ig1 domain has yet to be demonstrated.

Interaction of PST with NRP-2

Unlike NCAM and SynCAM 1, NRP-2 is polysialylated on O-linked glycans [293]. To determine the precise location of O-glycan polysialylation in NRP-2, other ongoing work from our laboratory has focused on a threonine-rich linker region located between the MAM and the second coagulation factor 5/8 type domains (see Chapter I, Figure 6). Using the OGPET V1.6 O-glycosylation electronic prediction tool and mutational analyses, several potential O-glycans within the Thr-rich linker were identified as potential sites of polysialylation and mutation of these residues did indeed decrease NRP-2 polysialylation (unpublished results).

The laboratory then decided to look for a region that may serve as a docking site for the polySTs, and decided to focus on the NRP-2 MAM domain. This decision was made taking into consideration the relative distance of this domain from the cell membrane and the location of the putative sites of polysialylation in the linker between the second coagulation factor 5/8 type domain and the MAM domain. The laboratory therefore modeled the NRP-2 MAM domain based on the structure of the receptor protein tyrosine phosphatase-μ MAM domain (PDB ID: 2C9A) [343]. We found two sets of acidic surfaces that form the halves of a β-sandwich within the MAM domain (Asp^{649}, Glu^{652}, Glu^{653}, Glu^{717}, Asp^{797}, and Asp^{798}; Glu^{735}, Glu^{739}, Asp^{765}, and Glu^{767}). As with SynCAM 1, recent mutational analyses suggest that several of these acidic residues are critical for effective NRP-2 polysialylation (unpublished data).

Based on these initial studies from the laboratory, I hypothesize that the NRP-2 MAM domain may function similarly as the NCAM FN1 and SynCAM 1 Ig2 domain, serving as a recognition and docking site for PST, allowing for the effective polysialylation of O-glycans in the linker region between the MAM and the second coagulation

factor 5/8 type domains (Figure 46, *"Predicted NRP-2-polyST interaction"*). Rationale for why STX may not polysialylate NRP-2 were discussed earlier in Chapter VI, but the patterns of the acidic residues found in the MAM domain may specifically prevent proper polysialylation by STX. Much more work is still needed to elucidate the NRP-2-polyST interaction.

Inherent differences in the mechanism of polysialylation of a particular substrate between PST and STX

Although both the PST and STX PBRs are critical for substrate polysialylation, it should be mentioned that these data presented here exemplify inherent differences within the polySTs themselves. For example, the recognition of NCAM is mediated by two PBR basic residues in PST, but is mediated by four in STX. Furthermore, recognition of SynCAM 1 by PST required definitive contributions by three PBR basic residues, whereas five STX PBR basic residues mediate this process with different levels of contribution. As pointed out previously, the PST PBR is critical for NRP-2 recognition, but the role of the PBR in the polysialylation of NRP-2 by STX provided inconsistent results. These findings suggest inherent differences in the precise biochemical mechanisms by which PST and STX polysialylate their substrates and suggest that the polySTs may prefer certain substrates over others.

This should not come as a surprise, as these data correlate with the hypothesis that protein polysialylation is a highly specific process. Furthermore, sequence alignment and secondary structure analyses shed some light on what these inherent difference could be. Upon lining up the amino acid sequences of PST and STX (see Chapter VI, Figure 35), I noticed that the region between the end of the transmembrane region and the start of the PBR in STX contained fifteen extra residues than PST, which constitutes almost all of the sixteen-residue difference between the two enzymes (PST is 359 residues, whereas STX in 375). Since these "extra" residues are prior to the PBR, it may be possible that there is slightly more space between the cell membrane and the PBR, which could position the STX PBR next to a particular substrate in a slightly different position than the PST PBR. Secondary structure prediction of the polyST PBRs using PSIPRED [327] indicated that there may be structural differences in the PBRs (data not shown). The PST PBR was predicted to be mainly helical in structure, whereas the STX PBR was a mix of helix, β-sheet, and coil. Structural studies are still needed to better understand the folds of the polyST PBRs.

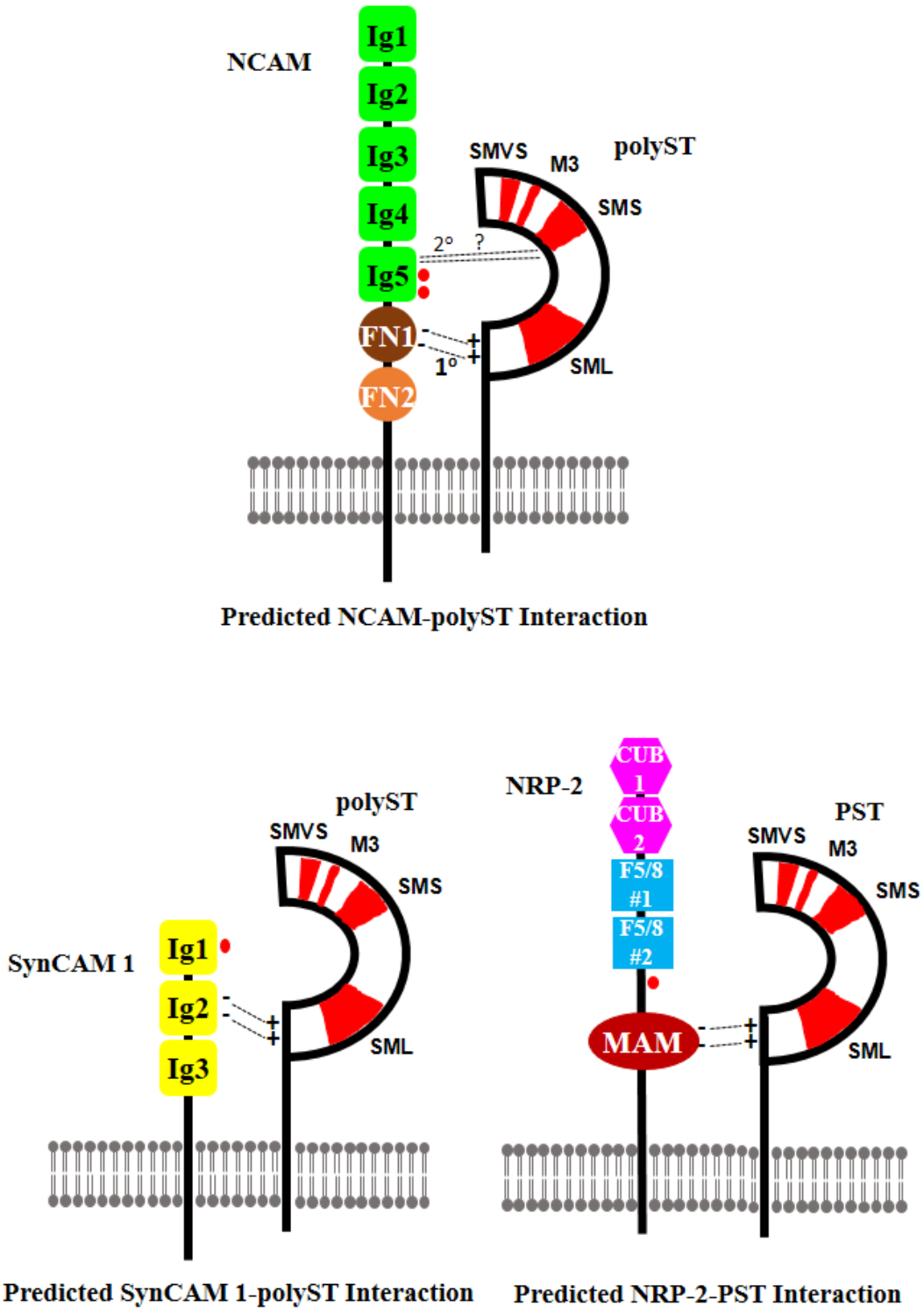

Figure 46. Predicted substrate interactions for polyST PBR basic residues. Based on structural analysis (NCAM) and homology modeling (SynCAM 1 and NRP-2), the polyST PBR region may form electrostatic interactions with acidic residues in the NCAM FN1, SynCAM 1 Ig2, and NRP-2 MAM domains. The approximate locations of substrate polysialylation are marked with a red dot. A secondary interaction involving NCAM Ig5 residues and unknown residues in the polyST is also predicted from previous studies [316].

Potential secondary polyST-substrate interaction near the location of polysialylation

Data from Thompson *et al.* [316] suggested that residues within the NCAM Ig5 domain may mediate a secondary interaction with the polySTs. What features of the polySTs could possibly mediate this interaction? At the current time, more work is needed to identify the entire secondary binding pocket, but we saw that replacing serine and asparagine residues in the Ig5 domain with large basic amino acids has a negative impact on the PST-NCAM interaction and NCAM polysialylation [316], and this might suggest that hydrogen bonds are important for this secondary interaction. One region to consider when looking for other potential residues within the polySTs that may mediate a second recognition site is the polysialyltransferase domain (PSTD) (Figure 47). This domain was initially identified by Nakata *et al.* [306], is 32 amino acids in length and consists of nine basic residues in PST (five lysines and four arginines) and seven basic residues in STX (three lysines and four arginines). Unlike the PBR, mutational analysis of select basic residues in the PSTD was shown to be critical for both substrate polysialylation and autopolysialylation, hence they are critical for overall polyST catalytic activity [305, 306]. However, only three PST PSTD basic residues were found to be critical for catalytic function (Arg^{252}, Lys^{276}, and Arg^{277}), thus this result does not exclude the possibility of additional electrostatic interactions or hydrogen bonds between other PSTD residues and residues in NCAM or other substrates.

Is it possible that an interaction between a polyST and a growing polySia chain also occur utilizing basic residues in the PSTD? PolySia is highly negatively charged, so a potential interaction between PSTD basic residues and polySia is plausible. In fact, this was the premise of Nakata *et al.* [306] as they searched for and then analyzed the PSTD of the polySTs. They reasoned that the PSTD interacts with a growing polySia chain, thus stabilizing and tethering the chain to the polyST and facilitating enzyme processivity. In accordance with this theory, the polyanionic heparin molecule was able to inhibit polySia synthesis by PST [306]. A potential polyST-polySia chain interaction would constitute a secondary (or tertiary) interaction and does not diminish the importance of the demonstrated polyST PBR-mediated protein-protein interaction between polyST and substrate that is required for recognition and polysialylation, or additional interactions between residues in the catalytic domain (including the

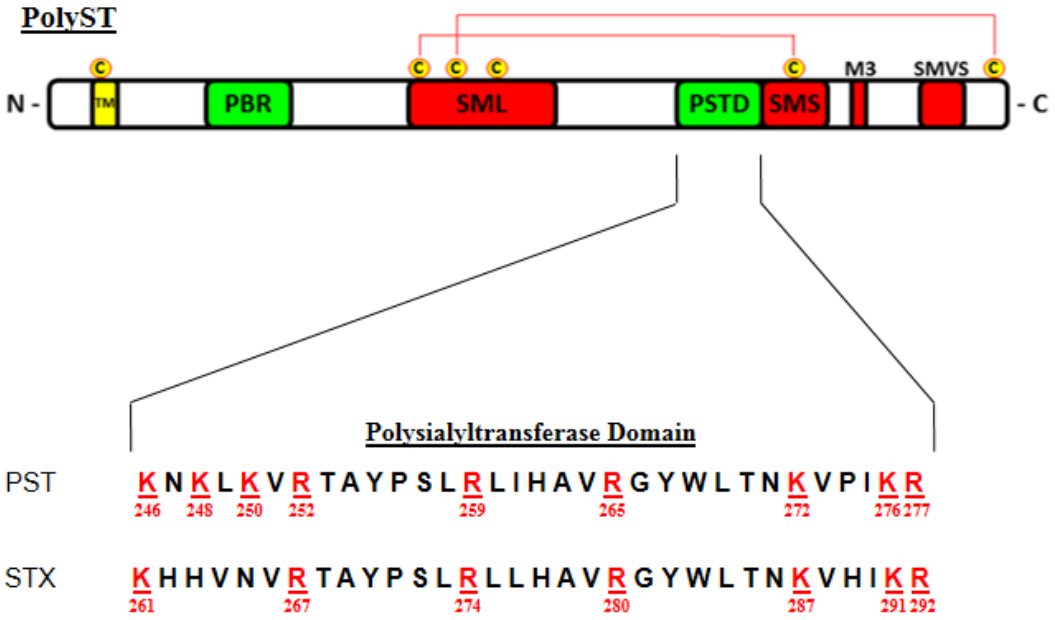

Figure 47. Sequence comparison of the PST and STX PSTDs. The PST and STX PSTD regions were aligned using Clustal W. The locations of all basic residues within these polyST PSTDs are highlighted in red and underlined.

PSTD) and the NCAM Ig5 domain that are required for proper polyST positioning and substrate polysialylation. Instead, it reflects the complexity of the polysialylation process.

Importance of structural analysis in understanding the biochemical mechanism of substrate polysialylation

In this work, I utilized a variety of biochemical techniques to decipher the mechanism of polyST-substrate interaction. What is currently lacking in the field is concrete structural analyses that could definitively show us where the potential polyST-substrate interactions are occurring and characterize the binding interface. In terms of NCAM, our laboratory has crystallized the Ig5-FN1 tandem, which represents one half of the interaction. However, at the current time, the crystal structures of the polySTs are unknown. Furthermore, homology modeling of the polySTs is not currently possible, as the only sialyltransferases crystallized to date, including bovine ST3Gal I [344], the CstII enzyme from *Campylobacter jejuni* [345], or a multifunctional sialyltransferase from *Pasturella multocida* [346], show very limited overall homology to the polySTs and no homology prior to the polyST large sialylmotif. Secondary structure predictions using PSIPRED [327] also do not give information as to the relative orientation of polyST PBR basic residue side chains, or their relative orientation to one another. Despite these current issues, structural studies are a key future direction to better understanding the interaction between polySTs and their substrates because these studies will aid tremendously in the overall long-term goal of this work—to find ways to alter the polyST-substrate interaction for therapeutic purposes.

Alongside the studies presented in this thesis work, I along with other members of the laboratory and others, have made attempts at preparing soluble PST or a PST PBR peptide together with our collaborator Dr. Kelley Moremen (Complex Carbohydrate Research Center, University of Georgia at Athens) in preparation for crystallization trials. At the current time, these studies are ongoing. We are also in ongoing collaboration with the University of Illinois at Chicago Center for Structural Biology at the current time to utilize ITC and NMR studies to quantify the polyST PBR interaction with NCAM Ig5-FN1 and also characterize the binding interface.

Final comments

The modification of substrates with polySia has many implications for human physiology, and without it, we cannot survive. PolySia has critical roles in the development of the nervous system, as well as in memory formation, olfaction, neuron repair, synapse formation, and immune cell chemotaxis. At the same time, too much polySia in the wrong places can also lead to death. This is true of several human tumors, in which polySia is abnormally upregulated in the later stages of cancers both within and outside of the nervous system. With polySia being both a beneficial and detrimental glycan, targeting the polyST-substrate interaction represents a possible therapeutic approach, especially in human disease. However, we cannot begin to know if this is even possible unless we understand the biochemical mechanism behind protein-specific polysialylation. It is for this reason that I undertook the studies presented in this thesis. Although there is still much more to be done, it is my hope that the work I presented here will contribute to a foundation of knowledge that can be built upon to one day create and utilize therapeutic inhibitors targeting the polyST-substrate interaction with the intent of contributing to the alleviation of human disease.

CITED LITERATURE

1. Stanley, P. (2011) Golgi Glycosylation. *Cold Spring Harb. Perspect. Biol*. **3**, 1-13.

2. Moremen, K.W., Tiemeyer, M., and Nairn, A.V. (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Bio*. **13**, 448-462.

3. Cummings, R.D. (2009) The repertoire of glycan determinants in the human glycome. *Mol. biosyst.* **5**, 1087-1104.

4. Nairn, A.V., York, W.S., Harris, K., Hall, E.M., Pierce, J.M., and Moremen, K.W. (2008) Regulation of glycan structures in animal tissues: transcript profiling of glycan-related genes. *J. Biol. Chem.* **283**, 17298-17313.

5. Lis, H., and Sharon, N. (1993) Protein glycosylation: structural and functional aspects. *Cur. J. Biochem.* **218**, 1-27.

6. Hounsell, E.F., Davies, M.J., and Renouf, D.V. (1996) O-linked protein glycosylation structure and function. *Glycoconjugate J.* **13**, 19-26.

7. Varki, A. (1993) Biological roles of the oligosaccharides: all of the theories are correct. *Glycobiology*. **3**, 97-130.

8. Roth, J., Zuber, C., Park, S., Jang, I., Lee, Y., Kysela, K.G., Le Fourn, V., Santimaria, R., Guhl, B., and Cho, J.W. (2010) Protein N-glycosylation, protein folding, and protein quality control. *Mol. Cells.* **30**, 497-506.

9. Hoseki, J., Ushioda, R., and Nagata, K. (2010) Mechanism and components of endoplasmic reticulum-associated degradation. *J. Biochem.* **147**, 19-25.

10. Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Bio.* **4**, 181-191.

11. Roth, J. (2002) Protein N-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions. *Biol. Cell.* **102**, 285-303.

12. Haltiwanger, R.S., and Lowe, J.B. (2004) Role of glycosylation in development. *Annu. Rev. Biochem.* **73**, 491-537.

13. Gu, J., Isaji, T., Xu, Q., Kariya, Y., Gu, W., Fukuda, T., and Du, Y. (2012) Potential roles of N-glycosylation in cell adhesion. *Glycoconj. J.* **29**, 599-607.

14. Fogel, A.I., Akins, M.R., Krupp, A.J., Stagi, M., Stein, V., and Biederer, T. (2007) SynCAMs organize synapses through heterophilic adhesion. *J. Neurosci.* **27**, 12516-12530.

15. Lien, E.C., Nagiec, M.J., and Dohlman, H.G. (2013) Proper protein glycosylation promotes mitogen-activated protein kinase signal fidelity. *Biochemistry.* **52**, 115-124.

16. Kosloski, M.P., Miclea, R.D., and Balu-lyer, L.D. (2009) Role of glycosylation in conformational stability, activity, macromolecular interaction and immunogenicity of recombinant human factor VIII. *AAPS J.* **11**, 424-431.

17. Markwell, M.A., and Paulson, J.C. (1980) Sendai virus utilizes specific sialyloligosaccharides as host cell receptor determinants. *Proc. Natl. Acad. Sci. USA*

18. Carroll, S.M., and Paulson, J.C. (1985) Differential infection of receptor-modified host cells by receptor-specific influenza viruses. *Virus Res.* **3**, 165-179.

19. Rogers, G.N., Daniels, R.S., Skehel, J.J., Wiley, D.C., Wang, X., Higa H.H., and Paulson, J.C. (1985) Host-mediated selection of influenza virus receptor variants. *J. Biol. Chem.* **260**, 7362-7367.

20. Rogers, G.N., Herrler, G., Paulson, J.C., and Klenk, H.-D. (1986) Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. *J. Biol. Chem.* **261**, 5947-5951.

21. Weis, W., Brown, J.H., Cusack, S., Paulson, J.C., Skehel, J.J., and Wiley, D.C. (1988) Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. *Nature.* **333**, 426-431.

22. Zingales, B., Carniol, C., De Lederkremer, R.M., and Colli, W. (1987) Direct sialic acid transfer from a protein donor to glycolipids of trypomastigote forms of Trypanosoma cruzi. *Mol. Biochem. Parasitol.* **26**, 135-144.

23. Libby, P., Alroy, J., and Pereira, M.E.A. (1989) A neuraminidase from Trypanosoma cruzi removes sialic acid from the surface of mammalian myocardial and endothelial cells. *J. Clin. Invest.* **77**, 127-135.

24. Schenkman, S., Jiang, M.-S., Hart, G.W., and Nussenzweig, V. (1991) A novel cell surface trans-sialidase of Trypanosoma cruzi generates a stage-specific epitope required for invasion of mammalian cells. *Cell*. **65**, 1117-1125.

25. Shayman, J.A., and Radin, N.S. (1991) Structure and function of renal glycosphingolipids. *Am. J. Phisiol.* **260**, F291-302.

26. Höök, M., Kjellén, L., and Johansson, S. (1984) Cell-surface glycosaminoglycans. *Annu. Rev. Biochem.* **53**, 847-869.

27. Rabinovich, G.A., van Kooyk, Y., and Cobb, B.A. (2012) Glycobiology of immune responses. *Ann. N.Y. Acad. Sci.* **1253**, 1-15.

28. Scott, D.W., and Patel, R.P. (2013) Endothelial heterogeneity and adhesion molecules N-glycosylation: Implications in leukocyte trafficking in inflammation. *Glycobiology.* In Press.

29. Buzás, E.I., György, B., Pásztói, M., Jelinek, I., Falus, A., and Gabius, H.J. (2006) Carbohydrate recognition systems in autoimmunity. *Autoimmunity.* **39**, 691-704.

30. Grigorian, A., Mkhikian, H., and Demetriou, M. (2012) Interleukin-2, interleukin-7, T cell-mediated autoimmunity, and N-glycosylation. *Ann. N.Y. Acad. Sci.* **1253**, 49-57.

31. Rambaruth, N.D., and Dwek, M.V. (2011) Cell surface glycan-lectin interactions in tumor metastasis. *Acta Histochem.* **113**, 591-600.

32. Hart, G.W., Slawson, C., Ramirez-Correa, G., and Lagerlof, O. (2011) Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annu. Rev. Biochem.* **80**, 825-858.

33. Lowe, J.B., and Marth, J.D. (2003) A genetic approach to mammalian glycan function. *Annu. Rev. Biochem.* **72**, 643-691.

34. Schnaar, R.L., Suzuki, A., and Stanley, P. (2009) Glycosphingolipids. In *Essentials of glycobiology* (ed. Varki, A., et al.), pp. 129-142. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

35. Ferguson, M.A. (1999) The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J. Cell. Sci.* **112**, 2799-2809.

36. Hart, G.W. (1999) In *Essentials of Glycobiology*. (ed. Varki, A., et al.), pp. 131-143. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

37. Schwartz, F., and Aebi, M. (2011) Mechanisms and principles of N-linked protein glycosylation. *Curr. Opin. Struc. Biol.* **21**, 576-582.

38. Durand, G., and Seta, N. (2000) Protein glycosylation and diseases: blood and urinary oligosaccharides as markers for diagnosis and therapeutic monitoring. *Clin. Chem.* **46**, 795-805.

39. Kornfeld, R., and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **57**, 631-634.

40. Nakayama, Y., Nakamura, N., Tsuji, D., Itoh, K., and Kurosaka, A. (2013) Genetic diseases associated with protein glycosylation disorders in mammals. In *Genetic Disorders*. (ed. Puiu, M.), pp. 243-269. InTech North America, New York, NY.

41. North, S.J., Huang, H.H., Sundaram, S., Jang-Lee, J., Etienne, A.T., Trollope, A., Chalabi, S., Dell, A., Stanley, P., and Haslam, S.M. (2010) Glycomics profiling of Chinese hamster ovary cell glycosylation mutants reveals N-glycans of a novel size and complexity. *J. Biol. Chem.* **285**, 5759-5775.

42. Surani, M.A.H. (1979) Glycoprotein synthesis and inhibition of glycosylation by tunicamycin in preimplantation mouse embryos: compaction and trophoblast adhesion. *Cell.* **18**, 217-227.

43. Marek, K.W., Vijay, I.K., and Marth, J.D. (1999) A recessive deletion in the GlcNAc-1-phosphotransferase gene results in pre-implantation embryonic lethality. *Glycobiology*. **9**, 1263-1271.

44. Sarkar, M., Hull, E., Nishikawa, Y., Simpson, R.J., Moritz, R.L., Dunn, R., and Schachter, H. (1991) Molecular cloning and expression of cDNA encoding the enzyme that controls conversion of high-mannose to hybrid and complex N-glycans: UDP-N-acetylglucosamine: alpha-3-D-mannosidase beta-1,2-N-acetylglucosaminyltransferase I. *Proc. Natl. Acad. Sci.* **88**, 234-238.

45. Layton, W.M. (1976) Random determination of a developmental process: reversal of normal visceral asymmetry in the mouse. *J. Hered.* **67**, 336-338.

46. Campbell, R.M., Metzler, M., Granovsky, M., Dennis, J.W., and Marth, J.D. (1995) Complex asparagine-linked oligosaccharides in MgatI-null embryos. *Glycobiology.* **5**, 535-543.

47. Moremen, K.W., and Robbins, P.W. (1991) Isolation, characterization, and expression of cDNAs encoding murine alpha-mannosidase II, a Golgi enzyme that controls conversion of high mannose to complex N-glycans. *J. Cell Biol.* **115**, 1521-1534.

48. Chui, D., Oh-Eda, M., Liao, Y.F., Paneerselvam, K., Lal, A., Marek, K.W., Freeze, H.H., Moremen, K.W., Fukuda, M.N., and Marth J.D. Alpha-mannosidase-II deficiency results in dyserythropoiesis and unveils and alternate pathway in oligosaccharide biosynthesis. *Cell.* **90**, 157-167.

49. Chui, D., Sellakumar, G., Green, R.S., Sutton-Smith, M., McQuistan, T., Marek, K., Morris, H., Dell, A., and Marth, J. (2001) Genetic remodeling of protein glycosylation in vivo induces autoimmune disease. *Proc. Natl. Acad. Sci.* **98**, 1142-1147.

50. Brockhausen, I., Schachter, H., and Stanley, P. (2009) In *Essentials of Glycobiology* (ed. Varki, A., et al), pp. 115-128. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

51. Bennett, E.P., Mandel, U., Clausen, H., Gerken, T.A., Fritz, T.A., and Tabak, L.A. (2012) Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. *Glycobiology.* **22**, 736-756.

52. Martin, P.T. (2003) Dystroglycan glycosylation and its role in matrix binding in skeletal muscle. *Glycobiology.* **13**, 55R-66R.

53. Nakamura, N., Lyalin, D., and Panin, V.M. (2010) Protein O-mannosylation in animal development and physiology: from human disorders to Drosophila phenotypes. *Semin. Cell Dev. Biol.* **21**, 622-630.

54. Luther, K.B., and Haltiwanger, R.S. (2009) Role of unusual O-glycans in intercellular signaling. *Int. J. Biochem. Cell Biol.* **41**, 1011-1024.

55. Leonhard-Melief, C., and Haltiwanger, R.S. (2010) O-fucosylation of thrombospondin type 1 repeats. *Methods Enzymol.* **480**, 401-416.

56. Wilson, I.B. (2004) The never-ending story of peptide O-xylosyltransferase. *Cell Mol. Life Sci.* **61**, 794-809.

57. Götting, C., Kuhn, J., and Kleesiek, K. (2007) Human xylosyltransferases in health and disease. *Cell Mol. Life Sci.* **64**, 1498-1517.

58. Yamauchi, M., and Sricholpech, M. (2012) Lysine post-translational modifications of collagen. *Essays Biochem.* **52**, 113-133.

59. Lomako, J., Lomako, W.M., and Whelan, W.J. (2004) Glycogenin: the primer for mammalian and yeast glycogen synthesis. *Biochim. Biophys. Acta.* **6**, 45-55.

60. Hakomori, S. (2002) The glycosynapse. *Proc. Natl. Acad. Sci.* **99**, 225-232.

61. Hang, H.C., and Bertozzi, C.R. (2005) The chemistry and biology of mucin-type O-linked glycosylation. *Bioorgan. Med. Chem.* **13**, 5021-5034.

62. Daley, W.P., Peters, S.B., and Larsen, M. (2008) Extracellular matrix dynamics in development and regenerative medicine. *J. Cell Sci.* **121**, 25-64.

63. Rozario, T., and DeSimone, D.W. (2010) The extracellular matrix in development and morphogenesis: a dynamic view. *Dev. Biol.* **341**, 126-140.

64. Tabak, L.A. (1995) In defense of the oral cavity: structure, biosynthesis and function of salivary mucins. *Annu. Rev. Physiol.* **57**, 547-564.

65. Rose, M.C., and Voynow, J.A. (2006) Respiratory tract mucin genes and mucin glycoproteins in health disease. *Physiol. Rev.* **86**, 245-278.

66. Roch, C., Kuhn, J., Kleesiek, K., and Gotting, C. (2010) Differences in gene expression of human xylosyltransferases and determination of acceptor specificities for various proteoglycans. *Biochem. Biophys. Res. Commun.* **391**, 685-691.

67. Ten Hagen, K.G., and Tran D. (2002) A UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase is essential for viability in Drosophila melanogaster. *J. Biol. Chem.* **277**, 22616-22622.

68. Schwientek, T., Bennett, E.P., Flores, C., Thacker, J., Hollmann, M., Reis, C.A., Behrens, J., Mandel, U., Keck, B., Schäfer, M.A., Haselmann, K., Zubarev, R., Roepstorff, P., Burchell, J.M., Taylor-Papadimitriou, J., Hollingsworth, M.A., and Clausen, H. (2002) Functional conservation of subfamilies of putative UDP-N-acetylgalactosamine:polypeptide N-acetylgalactosaminyltransferases in Drosophila, Caenorhabditis elegans, and mammals. *J. Biol. Chem.* **277**, 22623-22638.

69. Tian, E., and Ten Hagen, K.G. (2007) A UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase is required for epithelial tube formation. *J. Biol. Chem.* **282**, 606-614.

70. Lohmann, D.R., Buiting, K., Lüdecke, H.J., and Horsthemke, B. (1997) The murine Ext1 gene shows a high level of sequence similarity with its human homologue and is part of a conserved linkage group on chromosome 15. *Cytogenet. Cell Genet.* **76**, 164-166.

71. Lin, X., Wei, G., Shi, Z., Dryer, L., Esko, J.D., Wells, D.E., and Matzuk, M.M. (2000) Disruption of gastrulation and heparin sulfate biosynthesis in EXT1-deficient mice. *Dev. Biol.* **224**, 299-311.

72. Ringvall, M., Ledin, J., Holmborn, K., van Kuppevelt, T., Ellin, F., Eriksson, I., Olofsson, A.M., Kjellen, L., and Forsberg, E. (2000) Defective heparin sulfate biosynthesis and neonatal lethality in mice lacking N-deacetylase/N-sulfotransferase-1. *J. Biol. Chem.* **275**, 25926-25930.

73. Camenisch, T.D., Spicer, A.P., Brehm-Gibson, T., Biesterfeldt, J., Augustine, M.L., Calabro Jr, A., Kubalak, S., Klewer, S.E., and McDonald, J.A. (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J. Clin. Invest.* **106**, 349-360.

74. Butkinaree, C., Park, K., and Hart, G.W. (2010) O-linked β-N-acetylglucosamine (O-GlcNAc): Extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress. *Biochim. Biophys. Acta.* **1800**, 96-106.

75. Shafi, R., Iyer, S.P., Ellies, L.G., O'Donnell, N., Marek, K.W., Chui, D., Hart, G.W., and Marth, J.D. (2000) The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. *Proc. Natl. Acad. Sci. USA.* **23**, 5735-5739.

76. Wang, Z., Gucek, M., and Hart, G.W. (2008) Cross-talk between GlcNAcylation and phosphorylation: site specific phosphorylation dynamics in response to globally elevated O-GLcNAc. *Proc. Natl. Acad. Sci. USA.* **105**, 13793-13798.

77. Wang, Z., Pandey, A., and Hart, G.W. (2007) Dynamic interplay between O-linked N-acetylglucosaminylation and glycogen synthase kinase-3-dependent phosphorylation. *Mol. Cell Proteomics.* **6**, 1365-1379.

78. Wang, Z., Udeshi, N.D., Slawson, C., Compton, P.D., Sakabe, K., Cheung, W.D., Shabanowitz, J., Hunt, D.F., and Hart, G.W. (2010) Extensive crosstalk between O-GlcNAcylation and phosphorylation regulates cytokinesis. *Sci. Signal.* **3**, ra2.

79. Gong, C.X., and Igbal, K. (2008) Hyperphosphorylation of microtubule-associated protein tau: a promising therapeutic target for Alzheimer disease. *Curr. Med. Chem.* **15**, 2321-2328.

80. O'Donnell, N., Zachara, N.E., Hart, G.W., and Marth, J.D. (2004) Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. *Mol. Cell Biol.* **24**, 1680-1690.

81. Yuzwa, S.A., Macauley, M.S., Heinonen, J.E., Shan, X., Dennis, R.J., He, Y., Whitworth, G.E., Stubbs, K.A., McEachern, E.J., Davies, G.J., and Vocadlo, D.J. (2008) A potent mechanism-inspired O-GlcNAcase inhibitor that blocks phosphorylation of tau in vivo. *Nat. Chem. Biol.* **4**, 483-490.

82. Hanover, J.A., Lai, Z., Lee, G., Lubas, W.A., and Sato, S.M. (1999) Elevated O-linked N-acetylglucosamine metabolism in pancreatic beta-cells. *Arch. Biochem. Biophys.* **362**, 38-45.

83. Liu, K., Paterson, A.J., Chin, E., and Kudlow, J.E. (2000) Glucose promotes protein modification by O-linked GlcNAc in pancreatic beta cells: linkage of O-linked GlcNAc to beta cell death. *Proc. Natl. Acad. Sci. USA.* **14**, 2820-2850.

84. Parker, G.J., Lund, K.C., Taylor, R.P., and McClain, D.A. (2003) Insulin resistance of glycogen synthase mediated by o-linked N-acetylglucosamine. *J. Biol. Chem.* **278**, 10022-10027.

85. Vosseller, K., Wells, L., Daniel Lane, M., and Hart, G.W. (2002) Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA.* **99**, 5313-5318.

86. Kamemura, K., and Hart, G.W. (2003) Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: a new paradigm for metabolic control of signal transduction and transcription. *Prog. Nucleic Acid Res. Mol. Biol.* **73**, 107-136.

87. Wells, L., Slawson, C., and Hart, G.W. (2011) The E2F-1 associated retinoblastoma-susceptibility gene product is modified by O-GlcNAc. *Amino Acids.* **40**, 877-883.

88. Sayat, R., Leber, B., Grubac, V., Wiltshire, L., and Persad, S. (2008) O-GlcNAc-glycosylation of beta-catenin regulates its nuclear localization and transcriptional activity. *Exp. Cell Res.* **314**, 2774-2787.

89. Jiang, M.S., and Hart, G.W. (1997) A subpopulation of estrogen receptors are modified by O-linked N-acetylglucosamine. *J. Biol. Chem.* **272**, 2421-2428.

90. Cheng, X., Cole, R.N., Zaia, J., and Hart, G.W. (2000) Alternative O-glycosylation/O-phosphorylatioon of the murine estrogen receptor beta. *Biochemistry.* **39**, 11609-11620.

91. Jaeken, J., and Matthijs, G. (2001) Congenital disorders of glycosylation. *Annu. Rev. Genomics Hum. Genet.* **2**, 129-151.

92. Stibler, H., Blennow, G., Kristiansson, B., Lindehammer, H., and Hagberg, B. (1994) Carbohydrate-deficient glycoprotein syndrome: clinical expression in adults with a new metabolic disease. *J. Neurol. Neurosurg. Psychiatry.* **57**, 552-556.

93. Amato, A.A., and Griggs, R.C. (2011) Overview of the muscular dystrophies. *Handb. Clin. Neurol.* **101**, 1-9.

94. McNally, E.M., and Pytel, P. (2007) Muscle diseases: the muscular dystrophies. *Annu. Rev. Pathol. Mech. Dis.* **2**, 87-109.

95. Lapidos, K.A., Kakkar, R., and McNally, E.M. (2004) The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circ. Res.* **94**, 1023-1031.

96. Blake, D.J., Weir, A., Newey, S.E., and Davies, K.E. (2002) Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol. Rev.* **82**, 291-329.

97. Henry, M.D., and Campbell, K.P. (1999) Dystroglycan inside and out. *Curr. Opin. Cell Biol.* **11**, 602-607.

98. Dobyns, W.B., Pagon, R.A., Armstrong, D., Curry, C.J., Greenberg, F., Grix, A., Holmes, L.B., Laxova, R., Michels, V.V., and Robinow, M. (1989) Diagnostic criteria for Walker-Warburg syndrome. *Am. J. Med. Genet.* **32**, 195-210.

99. Cormand, B., Pihko, H., Bayes, M., Valanne, P., Santavuori, B., Talim, R., Gershoni-Baruch, A., Ahmad, H., van Bokhoven, H.G., Brunner, T., Voit, H., Topaloglu, W.B., Dobyns, W.B., and Lehesjoki, A.E. (2001) Clinical and genetic distinction between Walker-Warburg syndrome and muscle-eye-brain disease. *Neurology.* **56**, 1059-1069.

100. Brockington, M., Yuva, Y., Prandini, P., Brown, S.C., Torelli, S., Benson, M.A., Herrmann, R., Anderson, L.V., Bashir, R., Burgunder, J.M., Fallet, S., Romero, N., Fardeau, M., Straub, V., Storey, G., Pollitt, C., Richard, I., Sewry, C.A., Bushby, K., Voit, T., Blake, D.J., and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. *Hum. Mol. Genet.* **10**, 2851-2859.

101. Lamanna, W.C., Lawrence, R., Sarrazin, S., and Esko, J.D. (2011) Secondary storage of dermatan sulfate in Sanfilippo disease. *J. Biol. Chem.* **286**, 6955-6962.

102. Prinetti, A., Prioni, S., Chiricozzi, E., Schuchman, E.H., Chigorno, V., and Sonnino, S. (2011) Secondary alterations of sphingolipid metabolism in lysosomal storage diseases. *Neurochem. Res.* **36**, 1654-1668.

103. Walkley, S.U., and Vanier, M.T. (2009) Secondary lipid accumulation in lysosomal disease. *Biochim. Biophys. Acta.* **1793**, 726-736.

104. Pshezhetsky, A.V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M.A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L., and Potier, M. (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nat. Genet.* **15**, 316-320.

105. Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G., and d'Azzo, A. (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Gene Dev.* **10**, 3156-3169.

106. Dierks, T., Schlotawa, L., Frese, M.-A., Radhakrishnan, K., von Figura, K., and Schmidt, B. (2009) Molecular basis of multiple sulfatase deficiency, mucolipidosis II/III and Niemann-Pick C1 disease – lysosomal storage disorders caused by defects of non-lysosomal proteins. *Biochimica et. Biophysica Acta.* **1793**, 710-725.

107. Kornfeld, S., and Sly, W.S. (2001) I-cell disease and pseudo-Hurler polydystrophy: disorders of lysosomal enzyme phosphorylation and localization. In *The Metabolic and Molecular Bases of Inherited Disease, vol. III* (eds. Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D.), pp. 3469-3505. McGraw-Hill, New York, NY.

108. Storch, S., and Braulke, T. (2005) Transport of lysosomal enzymes. In *Lysosomes, Eurekah* (ed. Saftig, P.), pp. 17-26. Landes Bioscience, Springer Science and Business Media, New York, NY.

109. Hasilik, A., Waheed, A., and von Figura, K. (1981) Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP-N-acetylglucosamine; absence of the activity in I-cell fibroblasts. *Biochem. Biophys. Res. Commun.* **98**, 761-767.

110. Reitman, M.L., and Kornfeld, S. (1981) Lysosomal enzyme targeting; N-acetylglucosaminyl-phospho-transferase selectively phosphorylates native lysosomal enzymes. *J. Biol. Chem.* **256**, 11977-11980.

111. Waheed, A., Hasilik, A., and von Figura, K. (1981) Processing of the phosphorylated recognition marker in lysosomal enzymes; characterization and partial purification of a microsomal alpha-N-acetylglucosaminyl phosphodiesterase. *J. Biol. Chem.* **10**, 5717-5721.

112. Couso, R., Lang, L., Roberts, R.M., and Kornfeld, S. (1986) Phosphorylation of the oligosaccharide of uteroferrin by UDP-GlcNAc:glycoprotein N-acetylglucosamine-1-phos-phosphotransferases from rat liver, Acanthamoeba castellani, and Dictyostelium discoideum requires alpha 1,2-linked mannose residues. *J. Biol. Chem.* **261**, 6326-6331.

113. Kornfeld, R., Bao, M., Brewer, K., Noll, C., and Canfield, W.M. (1998) Purification and multimeric structure of bovine N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase. *J. Biol. Chem.* **273**, 23203-23210.

114. Rohrer, J., and Kornfeld, R. (2001) Lysosomal hydrolase mannose 6-phosphate uncovering enzyme resides in the trans-Golgi network. *Mol. Biol. Cell.* **12**, 1623-1631.

115. Ghosh, P., Dahms, N.M., and Kornfeld, S. (2003) Mannose 6-phosphate receptors: new twists in the tale. *Nat. Rev. Mol. Cell Biol.* **4**, 202-212.

116. Leroy, G., and DeMars, R.I. (1967) Mutant enzymatic and cytological phenotypes in cultured fibroblasts. *Science.* **157**, 804-806.

117. Bargal, R., Zeigler, M., Abu-Libdeh, B., Zuri, V., Mandel, H., Ben Neriah, Z., Stewart, F., Elcioglu, N., Hindi, T., LeMerrer, M., Bach, G., and Raas-Rothschild, A. (2006) When mucolipidosis III meets mucolipidosis II: GNPTA gene mutations in 24 patients. *Mol. Genet. Metabol.* **88**, 359-363.

118. Maeda, Y., Ide, T., Koike, M., Uchiyama, Y., and Kinoshita, T. (2008) GPHR is a novel anion channel critical for acidification and functions of the Golgi apparatus. *Nat. Cell Biol.* **10**, 1135-1145.

119. Maeda, Y., and Kinoshita, T. (2010) The acidic environment of the Golgi is critical for glycosylation and transport. *Methods Enzymol.* **480**, 495-510.

120. Sakaidani, Y., Ichiyanagi, N., Saito, C., Nomura, T., Ito, M., Nishio, Y., Nadano, D., Matsuda, T., Furukawa, K., and Okajima, T. (2012) O-linked-N-acetylglucosamine modification of mammalian Notch receptors by an atypical O-GlcNAc transferase Eogt1. *Biochem. Biophys. Res. Commun.* **419**, 14-19.

121. Shao, L., Moloney, D.J., and Haltiwanger, R.S. (2003) Fringe modifies O-fucose on mouse Notch1 at epidermal growth factor-like repeats within the ligand-binding site and the Abruptex region. *J. Biol. Chem.* **278**, 7775-7782.

122. Wang, Y., Shao, L., Shi, S., Harris, R.J., Spellman, M.W., Stanley, P., and Haltiwanger, R. (2001) Modification of epidermal growth factor-like repeats with O-fucose: molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *J. Biol. Chem.* **276**, 40338-40345.

123. Rana, N.A., and Haltiwanger, R.S. (2011) Fringe benefits: functional and structural impacts of O-glycosylation on the extracellular domain of Notch receptors. *Curr. Opin. Struct. Biol.* **21**, 583-589.

124. Yao, D., Huang, Y., Wang, W., Yan, Q., Wei, L., Xin, W., Gerson, S., Stanley, P., Lowe, J.B., and Zhou, L. (2011) Protein O-fucosyltransferase 1 (Pofut1) regulates lymphoid and myeloid homeostasis through modulation of Notch receptor ligand interactions. *Blood.* **117**, 5652-5662.

125. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R.S., and Panin, V.M. (2006) Protein O-fucosyltransferase 2 adds O-fucose to thrombospondin type 1 repeats. *J. Biol. Chem.* **281**, 9393-9399.

126. Hofsteenge, J., Huwiler, K.G., Macek, B., Hess, D., Lawler, J., Mosher, D.F., and Peter-Katalinic, J. (2001) C-mannosylation and O-fucosylation of the thrombospondin type 1 module. *J. Biol. Chem.* **276**, 6485-6498.

127. Du, J., Takeuchi, H., Leonhard-Melief, C., Shroyer, K.R., Dlugosz, M., Haltiwanger, R.S., and Holdener, B.C. (2010) O-fucosylation of throombospondin type 1 repeats restricts epithelial to mesenchymal transition (EMT) and maintains epiblast pluripotency during mouse gastrulation. *Dev. Biol.* **346**, 25-38.

128. Harduin-Lepers, A., Recchi, M.-A., and Delannoy, P. (1995) 1994, the year of sialyltransferases. *Glycobiology.* **5**, 741-758.

129. Galuska, S.P., Oltmann-Norden, I., Geyer, H., Weinhold, B., Kuchelmeister, K., Hildebrandt, H., Gerardy-Schahn, R., Geyer, R., and Muhlenhoff, M. (2006) Polysialic acid profiles of mice expressing variant allelic combinations of the polysialyltransferases ST8SiaII and ST8SiaIV. *J. Biol. Chem.* **281**, 31605-31615.

130. Livingston, B.D., Jacobs, J.L., Glick, M.C., and Troy, F.A. (1988) Extended polysialic acid chains (n > 55) in glycoproteins from human neuroblastoma cells. *J. Biol. Chem.* **263**, 9443-9448.

131. Angata, K. and Fukuda, M. (2003) Polysialyltransferases: major players in polysialic acid synthesis on the neural cell adhesion molecule. *Biochimie.* **85**, 195-206.

132. Curreli, S., Arany, Z., Gerardy-Schahn, R., Mann, D., and Stamatos, N.M. (2007) Polysialylated neuropilin-2 is expressed on the surface of human dendritic cells and modulates dendritic cell-T lymphocyte interactions. *J. Biol. Chem.* **282**, 30346-30356.

133. Galuska, S.P., Kaup, M., Rollenhagen, M., Oltmann-Norden, I., Hartmann, M., Weinhold, B., Gerardy-Schahn, R., Geyer, R., Muehlenhoff, M., and Geyer, H. (2010) Synaptic cell adhesion molecule SynCAM 1 is a target for polysialylation in postnatal mouse brain. *Proc. Natl. Acad. Sci. USA.* **107,** 10250-10255.

134. James, W.M. and Agnew, W.S. (1987) Multiple oligosaccharide chains in the voltage-sensitive Na channel from electrophorus electricus: evidence for α-2,8-linked polysialic acid. *Biochem. Bioph. Res. Co.* **148**, 817-826.

135. Yabe, U., Sato, C., Matsuda, T., and Kitajima, K. (2003) Polysialic acid in human milk: CD36 is a new member of mammalian polysialic acid-containing glycoprotein. *J. Biol. Chem.* **278**, 13875-13880.

136. Muhlenhoff, M., Eckhardt, M., Bethe, A., Frosch, M., and Gerardy-Schahn, R. (1996) Autocatalytic polysialylation of polysialyltransferase-1. *EMBO J.* **15**, 6943-6950.

137. Close, B.E. and Colley, K.J. (1998) *In Vivo* autopolysialylation and localization of the polysialyltransferases PST and STX. *J. Biol. Chem.* **273**, 34586-34593.

138. Sandi, C. (2004) Stress, cognitive impairment and cell adhesion molecules. *Nat. Rev. Neurosci.* **5**, 917-930.

139. Kiselyov, V.V., Soroka, V., Berezin, V., and Bock, E. (2005) Structural biology of NCAM homophilic binding and activation of FGFR. *J. Neurochem.* **94**, 1169-1179.

140. Rao, Y., Wu, X.F., Gariepy, J., Rutishauser, U., and Siu, C.H. (1992) Identification of a peptide sequence involved in homophilic binding in the neural cell adhesion molecule NCAM. *J. Cell Biol.* **118**, 937-949.

141. Rao, Y., Wu, X.F., Yip, P., Gariepy, J., and Siu, C.H. (1993) Structural characterization of a homophilic binding site in the neural cell adhesion molecule. *J. Biol. Chem.* **268**, 630-638.

142. Zhou, H., Fuks, A., Alcaraz, G., Bolling, T.J., and Stanners, C.P. (1993) Homophilic adhesion between Ig superfamily carcinoembryonic antigen molecules involves double reciprocal bonds. *J. Cell Biol.* **122**, 951-960.

143. Ranheim, T.S., Edelman, G.M., and Cunningham, B.A. (1996) Homophilic adhesion mediated by the neural cell adhesion molecule involves multiple immunoglobulin domains. *Proc. Natl. Acad. Sci. USA.* **93**, 4071-4075.

144. Kiselyov, V.V., Berezin, V., Maar, T.E., Soroka, V., Edvardsen, K., Schousboe, A., and Bock, E. (1997) The first immunoglobulin-like neural cell adhesion molecule (NCAM) domain is involved in double-reciprocal interaction with the second immunoglobulin-like NCAM domain and in heparin binding. *J. Biol. Chem.* **272**, 125-134.

145. Atkins, A.R., Osborne, M.J., Lashuel, H.A., Edelman, G.M., Wright, P.E., Cunningham, B.A., and Dyson, H.J. (1999) Association between the first two immunoglobulin-like domains of the neural cell adhesion molecule N-CAM. *FEBS Lett.* **451**, 162-168.

146. Jensen, P.H., Soroka, V., Thomsen, N.K., Ralets, I., Berezin, V., Bock, E., and Poulsen, F.M. (1999) Structure and interactions of NCAM modules 1 and 2, basic elements in neural cell adhesion. *Natl. Struct. Biol.* **6**, 486-493.

147. Johnson, C.P., Fujimoto, I., Perrin-Tricaud, C., Rutishauser, U., and Leckband, D. (2004) Mechanism of homophilic adhesion by the neural cell adhesion molecule: use of multiple domains and flexibility. *Proc. Natl. Acad. Sci. USA.* **101**, 6963-6968.

148. Soroka, V., Kolkova, K., Kastrup, J.S., Diederichs, K., Breed, J., Kiselyov, V.V., Poulsen, F.M., Larsen, I.K., Welte, W., Berezin, V., Bock, E., and Kasper, C. (2003) Structure and interactions of NCAM Ig1-2-3- suggest a novel zipper mechanism for homophilic adhesion. *Structure.* **11**, 1291-1301.

149. Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R., and Edelman, G.M. (1987) Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science.* **15**, 799-806.

150. Nelson, R.W., Bates, P.A., and Rutishauser, U. (1995) Protein determinants for specific polysialylation of the neural cell adhesion molecule. *J. Biol. Chem.* **270**, 17171-17179.

151. Rutishauser, U. (2008) Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. *Nature.* **9**, 26-35.

152. Fujimoto, I., Bruses, J.L., and Rutishauser, U. (2001) Regulation of cell adhesion by polysialic acid: effects on cadherin, immunoglobulin cell adhesion molecule, and integrin function and independence from neural cell adhesion molecule binding or signaling activity. *J. Biol. Chem.* **276**, 31745-31751.

153. Horstkorte, R., Lebner, N., Gerardy-Schahn, R., Lucka, L., Danker, K., and Reutter, W. (1999) Expression of the Polysialyltransferase ST8SiaIV: Polysialylation interferes with adhesion of PC12 cells *in Vitro*. *Exp. Cell Res.* **246**, 122-128.

154. Johnson, C.P., Fujimoto, I., Rutishauser, U., and Leckband, D.E. (2005) Direct evidence that neural cell adhesion molecule (NCAM) polysialylation increases intermembrane repulsion and abrogates adhesion. *J. Biol. Chem.* **280**, 137-145.

155. Conchonaud, F., Nicolas, S., Amoureux, M.-C., Menager, C., Marguet, D., Lenne, P.-F., Rougon, G., and Matarazzo, V. (2007) Polysialylation increases lateral diffusion of neural cell adhesion molecule in the cell membrane. *J. Biol. Chem.* **282**, 26266-26274.

156. Ditlevsen, D.K., Povlsen, G.K., Berezin V., and Bock, E. (2008) NCAM-induced intracellular signaling revisited. *J. Neurosci. Res.* **86**, 727-743.

157. Beggs, H.E., Soriano, P., and Maness, P.F. (1994) NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice. *J. Cell Biol.* **127**, 825-833.

158. Beggs, H.E., Baragona, S.C., Hemperly, J.J., and Maness, P.F. (1997) NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). *J. Biol. Chem.* **272**, 8310-8319.

159. Kramer, E.M., Klein, C., Koch, T., Boytinck, M., and Trotter, J. (1999) Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. *J. Biol. Chem.* **274**, 29042-29049.

160. Bodrikov, V., Leshchynska, I., Sytnyk, V., Overvoorde, J., dan Hertog, J., and Schachner, M. (2005) RPTPalpha is essential for NCAM-mediated p59fyn activation and neurite elongation. *J. Cell Biol.* **168**, 127-139.

161. Kiselyov, V.V., Skladchikova, G., Hinsby, A.M., Jensen, P.M., Kulahin, N., Soroka, V., Pedersen, N., Tsetlin, V., Poulsen, F.M., Berezin, V., and Bock, E. (2003) Structural basis for a direct interaction between FGFR1 and NCAM and evidence for a regulatory role of ATP. *Structure.* **11**, 691-701.

162. Saffell, J.L., Williams, E.J., Mason, I.J., Walsh, F.S., and Doherty, P. (1997) Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. *Neuron.* **18**, 231-242.

163. Neiiendam, J.L, Kohler, L.B., Christensen, C., Li, S., Pedersen, M.V., Ditlevsen, D.K., Kornum, M.K., Kiselyov, V.V., Berezin, V., Bock, E. (2004) An NCAM-derived FGF-receptor agonist, the FGL-peptide, induces neurite outgrowth and neuronal survival in primary rat neurons. *J. Neurochem.* **91**, 920-935.

164. Kolkova, K., Novitskaya, V., Pedersen, N., Berezin, V., and Bock, E. (2000) Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. *J. Neurosci.* **20**, 2238-2246.

165. Williams, E.J., Furness, J., Walsh, F.S., and Doherty, P. (1994) Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron.* **13**, 583-594.

166. Hinsby, A.M., Lundfald, L., Ditlevsen, D.K., Korshunova, I., Juhl, L., Meakin, S.O., Berezin, V., and Bock, E. (2004) ShcA regulates neurite outgrowth stimulated by neural cell adhesion molecule but not by fibroblast growth factor 2: evidence for a distinct fibroblast growth factor receptor response to neural cell adhesion molecule activation. *J. Neurochem.* **91**, 694-703.

167. Acheson, A., Conover, J.C., Fandl, J.P., DeChiara, T.M., Russell, M., Thadani, A., Squinto, S.P., Yancopoulos, G.D., and Lindsay, R.M. (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature*. **374**, 450-453.

168. Huang, E.J., and Reichardt, L.F. (2001) Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24**, 677–736.

169. Ghosh, A., Carnahan, J., and Greenberg, M.E. (1994) Requirement for BDNF inactivity-dependent survival of cortical neurons. *Science*. **263**, 1618-1623.

170. Ernfors, P., Kucera, J., Lee, K.F., Loring, J., and Jaenisch, R. (1995) Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. *Int. J. Dev. Biol.* **39**, 799–807.

171. Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J.I., Goldin, A., Izquierdo, I., and Medina, J.H. (2008) BDNF is essential to promote persistence of long-term memory storage. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 2711–2716.

172. Vutskits, L., Djebbara-Hannas, Z., Zhang, H., Paccaud, J.P., Durbec, P., Rougon, G., Muller, D., and Kiss, J.Z. (2001) PSA-NCAM modulates BDNF-dependent survival and differentiation of cortical neurons. *Eur. J. Neurosci.* **13**, 1391-1402.

173. Lee, J. C., Mayer-Proschel, M., and Rao, M. S. (2000) Gliogenesis in the central nervous system. *Glia.* **30**, 105-121.

174. Blakemore, W. F., and Keirstead, H. S. (1999) The origin of remyelinating cells in the central nervous system. *J. Neuroimmunol.* **98**, 69-76.

175. Zhang, S. C., Ge, B., and Duncan, I. D. (1999) Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity. *Proc. Natl. Acad. Sci. USA.* **96**, 4089-4094.

176. Armstrong, R. C., Harvath, L., and Dubois-Dalcq, M. E. (1990) Type 1 astrocytes and oligodendrocyte-type 2 astrocyte glial progenitors migrate toward distinct molecules. *J. Neurosci. Res.* **27**, 400-407.

177. Zhang, H., Vutskits, L., Calaora, V., Durbec, P., and Kiss, J.Z. (2004) A role for the polysialic acid-neural cell adhesion molecule in PDGF-induced chemotaxis of oligodendrocyte precursor cells. *J. Cell Sci.* **117**, 93-103.

178. Glaser, T., Brose, C., Franceschini, I., Hamann, K., Smorodchenko, A., Zipp, F., Dubois-Dalcq, M., and Brustle, O. (2007) Neural cell adhesion molecule polysialylation enhances the sensitivity of embryonic stem cell-derived neural precursors to migration guidance cues. *Stem Cells.* **25**, 3016-3025.

179. Schmid, R.S., Graff, R.D., Schaller, M.D., Chen, S., Schachner, M., Hemperly, J.J., and Maness, P.F. (1999) NCAM stimulates the Ras-MAPK pathway and CREB phosphorylation in neuronal cells. *J. Neurobiol.* **38**, 542–558.

180. Seidenfaden, R., Krauter, A., Schertzinger, F., Gerardy-Schahn, R., and Hildebrandt, H. (2003) Polysialic acid directs tumor cell growth by controlling heterotrophic neural cell adhesion molecule interactions. *Mol. Cell Biol.* **23**, 5908-5918.

181. Gomez, T.M., and Spitzer, N.C. (2000) Regulation of growth cone behavior by calcium: new dynamics to earlier perspectives. *J. Neurobiol.* **44**, 174-183.

182. Doherty, P., Ashton, S.V., Moore, S.E., and Walsh, F.S. (1991) Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca^{2+} channels. *Cell.* **67**, 21-33.

183. Ronn, L.C., Dissing, S., Holm, A., Berezin, V., and Bock, E. (2002) Increased intra-cellular calcium is required for neurite outgrowth induced by a synthetic peptide ligand of NCAM. *FEBS Lett*. **518**, 60-66.

184. Kiryushko, D., Korshunova, I., Berezin, V., and Bock, E. (2006) Neural cell adhesion molecule induces intracellular signaling via multiple mechanisms of Ca^{2+} homeostasis. *Mol. Biol. Cell.* **17**, 2278-2286.

185. Kolkova, K., Stensman, H., Berezin, V., Bock, E., and Larsson, C. (2005) Distinct roles of PKC isoforms in NCAM-mediated neurite outgrowth. *J. Neurochem*. **92**, 886-894.

186. Ditlevsen, D.K., Kohler, L.B., Pedersen, M.V., Risell, M., Kolkova, K., Meyer, M., Berezin, V., and Bock, E. (2003) The role of phosphatidylinositol 3-kinase in neural cell adhesion molecule-mediated neuronal differentiation and survival. *J. Neurochem*. **84**, 546-556.

187. Ditlevsen, D.K., Kohler, L.B., Berezin, V., and Bock, E. (2007) Cyclic guanosine monophosphate signalling pathway plays a role in neural cell adhesion molecule-mediated neurite outgrowth and survival. *J. Neurosci. Res.* **85**,703-711.

188. Ono, S., Hane, M., Kitajima, K., and Sato, C. (2012) Novel regulation of fibroblast growth factor 2 (FGF2)-mediated cell growth by polysialic acid. *J. Biol. Chem.* **287**, 3710-3722.

189. Isomura, R., Kitajima, K., and Sato, C. (2011) Structural and functional impairments of polysialic acid by a mutated polysialyltransferase found in schizophrenia. *J. Biol. Chem.* **286**, 21535-21545.

190. Kanato, Y., Kitajima, K., and Sato, C. (2008) Direct binding of polysialic acid to a brain-derived neurotrophic factor depends on the degree of polymerization. *Glycobiology*. **18**, 1044-1053.

191. Hildebrandt, H., Muhlenhoff, M., Weinhold, B., and Gerardy-Schahn, R. (2007) Dissecting polysialic acid and NCAM functions in brain development. *J. Neurochem.* **103**, 56-64.

192. Seki, T. and Arai, Y. (1993) Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. *Neurosci. Res.* **17**, 265-290.

193. Bonfanti, L. (2006) PSA-NCAM in mammalian structural plasticity and neurogenesis. *Prog. Neurobiol.* **80**, 129-164.

194. Heckmat, A., Bitter-Suermann, D., and Schachner, M. (1990) Immunocytological localization of the highly polysialylated form of the neural cell adhesion molecule during development of the murine cerebellar cortex. *J. Comp. Neurol.* **291**, 457-467.

195. Bartsch, U., Kirchoff, F., and Schachner, M. (1990) Highly sialylated N-CAM is expressed in adult mouse optic nerve and retina. *J. Neurocytol.* **19**, 550-565.

196. Rutishauser, U. (2008) Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. *Nat. Rev. Neurosci.* **9**, 26-35.

197. Nakayama, J., Angata, K., Ong, E., Katsuyama, T., and Fukuda, M. (1998) Polysialic acid, a unique glycan that is developmentally regulated by two polysialyltransferases, PST and STX, in the central nervous system: from biosynthesis to function. *Pathol. Int.* **48**, 665-677.

198. Weinhold, B., Seidenfaden, R., Rockle, I., Muhlenhoff, M., Schertzinger, F., Conzelmann, S., Marth, J.D., Gerardy-Schahn, R., and Hildebrandt, H. (2005) Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. *J. Biol. Chem.* **280**, 42971-42977.

199. Hu, H., Tomasiewicz, H., Magnuson, T., and Rutishauser, U. (1996) The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. *Neuron.* **16**, 735-743.

200. Doyle, E., Nolan, P.M., Bell, R., and Regan, C.M. (1992) Hippocampal NCAM180 transiently increases sialylation during the acquisition and consolidation of a passive avoidance response in the adult rat. *J. Neurosci. Res.* **31**, 513-523.

201. Ni Dhuill, C.M., Fox, G.B., Pittock, S.J., O'Connell, A.W., Murphy, K.J., and Regan, C.M. (1999) Polysialylated neural cell adhesion molecule expression in the dentate gyrus of the human hippocampal formation from infancy to old age. *J. Neurosci. Res.* **55**, 99-106.

202. Stoenica, L., Senkov, O., Gerardy-Schahn, R., Weinhold, B., Schachner, M., and Dityatev, A. (2006) *In vivo* synaptic plasticity in the dentate gyrus of mice deficient in the neural cell adhesion molecule NCAM or its polysialic acid. *Eur. J. Neurosci.* **23**, 2255-2264.

203. Burgess, A., Wainwright, S.R., Shihabuddin, L.S., Rutishauser, U., Seki, T., and Aubert, I. (2008) Polysialic acid regulates the clustering, migration, and neuronal differentiation of progenitor cells in the adult hippocampus. *Dev. Neuobiol.* **68**, 1580-1590.

204. Becker, C.G., Artola, A., Gerardy-Schahn, R., Becker, T., Welzl, H., and Schachner, M. (1996) The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation. *J. Neurosci. Res.* **45**, 143-152.

205. Bliss, T.V., and Collingridge, G.L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature.* **361**, 31-39.

206. Cooke, S.F., and Bliss, T.V. (2006) Plasticity in the human central nervous system. *Brain.* **129**, 1659-1673.

207. Dityatev, A., Dityateva, G., Sytnyk, V., Delling, M., Toni, N., Nikonenko, I., Muller, D., and Schachner, M. (2004) Polysialylated neural cell adhesion molecule promotes remodeling and formation of hippocampal synapses. *J. Neurosci.* **24**, 9372-9382.

208. Curtis, M.A., Monzo, H.J., and Faull, R.L.M. (2009) The rostral migratory stream and olfactory system: smell, disease and slippery cells. *Prog. Brain Res.* **175**, 33-42.

209. Hu, H. (2000) Polysialic acid regulates chain formation by migrating olfactory neuron precursors. *J. Neurosci. Res.* 61:480-492.

210. Theodosis, D. T., Rougon, G., and Poulain, D. A. (1991) Retention of embryonic features by an adult neuronal system capable of plasticity: polysialylated neural cell adhesion molecule in the hypothalamo-neurohypophysial system. *Proc. Natl. Acad. Sci. USA.* **88**, 5494-5498

211. Kaur, G., Heera, P. K., and Srivastava, L. K. (2002) Neuroendocrine plasticity in GnRH release during rat estrous cycle: correlation with molecular markers of synaptic remodeling. *Brain Res.* **954**, 21-31.

212. Hoyk, Z., Parducz, A., and Theodosis, D. T. (2001) The highly sialylated isoform of the neural cell adhesion molecule is required for estradiol-induced morphological synaptic plasticity in the adult arcuate nucleus. *Eur. J. Neurosci.* **13**, 649-656.

213. Nothias, F., Vernier, P., von Boxberg, Y., Mirman, S., and Vincent, J. D. (1997) Modulation of NCAM polysialylation is associated with morphofunctional modifications in the hypothalamo-neurohypophysial system during lactation. *Eur. J. Neurosci.* **9**, 1553-1565.

214. Theodosis, D. T., Bonhomme, R., Vitiello, S., Rougon, G., and Poulain, D. A. (1999) Cell surface expression of polysialic acid on NCAM is a prerequisite for activity-dependent morphological neuronal and glial plasticity. *J. Neurosci.* **19**, 10228-10236.

215. Monlezun, S., Ouali, S., Poulain, D. A., and Theodosis, D. T. (2005) Polysialic acid is required for active phases of morphological plasticity of neurosecretory axons and their glia. *Mol. Cell. Neurosci.* **29**, 516-524.

216. Glass, J. D., Watanabe, M., Fedorkova, L., Shen, H., Ungers, G., and Rutishauser, U. (2003) Dynamic regulation of polysialylated neural cell adhesion molecule in the suprachiasmatic nucleus. *Neuroscience.* **117**, 203-211.

217. Glass, J. D., Shen, H., Fedorkova, L., Chen, L., Tomasiewicz, H., and Watanabe, M. (2000) Polysialylated neural cell adhesion molecule modulates photic signaling in the mouse suprachiasmatic nucleus. *Neurosci. Lett.* **280**, 207-210.

218. Prosser, R. A., Rutishauser, U., Ungers, G., Fedorkova, L., and Glass, J. D. (2003) Intrinsic role of polysialylated neural cell adhesion molecule in photic phase resetting of the mammalian circadian clock. *J. Neurosci.* **23**, 652-658.

219. Dusart, I., and Sotelo, C. (1994) Lack of Purkinje cell loss in adult rat cerebellum following protracted axotomy: degenerative changes and regenerative attempts of the severed axons. *J. Comp. Neurol.* **347**, 211-232.

220. Dusart, I., Morel, M.P., Wehrle, R., and Sotelo, C. (1999) Late axonal sprouting of injured Purkinje cells and its temporal correlation with permissive changes in the glial scar. *J. Comp. Neurol.* **408**, 399-418.

221. Boisseau, S., Nedelec, J., Poirier, V., Rougon, G., and Simonneau, M. (1991) Analysis of high PSA N-CAM expression during mammalian spinal cord and peripheral nervous system development. *Development*. **112**, 69-82.

222. Rutishauser, U., Watanabe, M., Silver, J., Troy, F.A., and Vimr, E.R. (1985) Specific alteration of NCAM-mediated cell adhesion by an endoneuraminidase. *J. Cell Biol.* **101**, 1842-1849.

223. Charter, N.W., Mahal, L.K., Koshland Jr., D.E., and Bertozzi, C.R. (2002) Differential effects of unnatural sialic acids on the polysialylation of the neural cell adhesion molecule and neuronal behavior. *J. Biol. Chem.* **277**, 9255-9261.

224. Thomaidou, D., Coquillat, D., Meintanis, S., Noda, M., Rougon, G., and Matsas, R. (2001) Soluble forms of NCAM and F3 neuronal cell adhesion molecules promote Schwann cell migration: identification of protein tyrosine phosphatases zeta/beta as the putative F3 receptors on Schwann cells. *J. Neurochem.* **78**, 767-778.

225. Lavdas, A.A., Franceschini, I., Dubois-Dalcq, M., and Matsas, R. (2006) Schwann cells genetically engineered to express PSA show enhanced migratory potential without impairment of their myelinating ability in vitro. *Glia*. **53**, 868-878.

226. Daniloff, J.K., Levi, G., Grumet, M., Rieger, F., and Edelman, G.M. (1986) Altered expression of neuronal cell adhesion molecules induced by nerve injury and repair. *J. Cell Biol.* **103**, 929-945.

227. Oumesmar, B.N., Vignais, L., Duhamel-Clerin, E., Avellana-Adalid, V., Rougon, G., and Baron-Van Evercooren, A. (1995) Expression of the highly polysialylated neural cell adhesion molecule during postnatal myelination and following chemically induced demyelination of the adult mouse spinal cord. *Eur. J. Neurosci.* **7**, 480-491.

228. Zhang, Y., Campbell, G., Anderson, P.N., Martini, R., Schachner, M., and Lieberman, A.R. (1995) Molecular basis of interactions between regenerating adult rat thalamic axons and Schwann cells in peripheral nerve grafts I. Neural cell adhesion molecules. *J. Comp. Neurol.* **361**, 193-209.

229. Jungnickel, J., Brämer, ,C., Bronzlik, P., Lipokatic-Takacs, E., Weinhold, B., Gerardy-Schahn, R., and Grothe, C. (2009) Level and localization of polysialic acid is critical for early peripheral nerve regeneration. *Mol. Cell Neurosci.* **40**, 374-381.

230. Carlstedt, T. (1985) Regenerating axons form nerve terminals at astrocytes. *Brain Res.* **34,** 188-191.

231. Liuzzi, F.J., and Lasek, R.J. (1987) Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway. *Science.* **237**, 642-645.

232. Chong, M.S., Woolf, C.J., Haque, N.S., and Anderson, P.N. (1999) Regeneration of axons from injured dorsal roots into the spinal cord in adult rats. *J. Comp. Neurol.* **410**, 42-54.

233. Pindzola, R.R., Doller, C., and Silver, J. (1993) Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. *Dev. Biol.* **156**, 34-48.

234. Zhang, Y., Tohyama, K., Winterbottom, J.K., Haque, N.S., Schachner, M., Lieberman, A.R., and Anderson, P.M. (2001) Correlation between putative inhibitory molecules at the dorsal root entry zone and failure of dorsal root axonal regeneration. *Mol. Cell Neurosci.* **17**, 444-459.

235. Beggah, A.T., Dours-Zimmermann, M.T., Barras, F.M., Brosius, A., Zimmermann, D.R., and Zurn, A.D. (2005) Lesion-induced differential expression and cell association of Neurocan, Brevican, Versican V1 and V2 in the mouse dorsal root entry zone. *Neuroscience.* **133**, 749-762.

236. Zhang, X., Bo, X., Anderson, P.N., Lieberman, A.R., and Zhang, Y. (2006) Distribution and expression of tissue inhibitors of metalloproteinase in dorsal root entry zone and dorsal column after dorsal root injury. *J. Neurosci. Res.* **84**, 278-290.

237. Ghosh, M., Tuesta, L.M., Puentes, R., Patel, S., Melendez, K., El Maarouf, A., Rutishauser, U., and Pearse, D.D. (2012) Extensive cell migration, axon regeneration, and improved function with polysialic acid-modified Schwann cells after spinal cord injury. *Glia.* **60**, 979-992.

238. Zhang, Y., Zhang, X., Wu, D., Verhaagen, J., Richardson, P.M., Yeh, J., and Bo, X. (2007) Lentiviral-mediated expression of polysialic acid in spinal cord and conditioning lesion promote regeneration of sensory axons into spinal cord. *Mol. Ther.* **15**, 1796-1804.

239. Zhang, Y., Ghadiri-Sani, M., Zhang, X., Richardson, P.M., Yeh, J., and Bo, X. (2007) Induced expression of polysialic acid in the spinal cord promotes regeneration of sensory axons. *Mol. Cell Neurosci.* **35**, 109-119.

240. Mehanna, A., Jakovcevski, I., Acar, A., Xiao, M., Loers, G., Rougon, G., Irintchev, A., and Schachner, M. (2010) Polysialic acid glycomimetic promotes functional recovery and plasticity after spinal cord injury in mice. *Mol. Ther.* **18**, 34-43.

241. Duveau, V., Arthaud, S., Rougier, A., and Le Gal La Salle, G. (2007) Polysialylation of NCAM is upregulated by hyperthermia and participates in heat shock preconditioning-induced neuroprotection. *Neurobiol. Dis.* **26**, 385-395.

242. Rejdak, R., Rejdak, K., Sieklucka-Dziuba, M., Stelmasiak, Z., and Grieb, P. (2001) Brain tolerance and preconditioning. *Pol. J. Pharmacol.* **53**, 73-79.

243. Pham, K., Nacher, J., Hof, P.R., and McEwen, B.S. (2003) Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. *Eur. J. Neurosci.* **17**, 879-886.

244. Roberts, G.W. (1991) Schizophrenia: a neuropathological perspective. *Br. J. Psychiatry.* **158**, 8-17.

245. Bloom, F.E. (1993) Advancing a neurodevelopmental origin for schizophrenia. *Arch. Gen. Psychiatry.* **50**, 224-227.

246. Conrad, A.J., Abebe, T., Austin, R., Forsythe, S., and Scheibel, B. (1991) Hippocampal pyramidal cell disarray in schizophrenia as a bilateral phenomenon. *Arch. Gen. Psychiatry.* **48**, 413-417.

247. Akbarian, S., Vinuela, A., Kim, J.J., Potkin, S.G., Bunney, W.E., and Jones, E.G. (1993) Distorted distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase neurons in temporal lobe of schizophrenics implies anomalous cortical development. *Arch. Gen. Psychiatry.* **50**, 178-187.

248. Akbarian, S., Bunney, W.E., Potkin, S.G., Wigal, S.B., Hagman, J.O., Sandman, C.A., and Jones, E.G. (1993) Altered distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase cells in frontal lobe of schizophrenics implies disturbances of cortical development. *Arch. Gen. Psychiatry.* **50**, 169-177.

249. Kotrla, K.J., and Weinberger, D.R. (1994) In *Schizophrenia: Exploring the Spectrum of Psychosis.* (ed. Ancill, R.J., Holliday, S., and Higenbottam, J.) pp. 67-94. Wiley, Chichister, U.K.

250. Morriss, R., Vinjamuri, I., Faizal, M.A., Bolton, C.A., and McCarthy, J.P. (2013) Training to recognize the early signs of recurrence in schizophrenia. *Cochrane Database Syst. Rev.* In Press.

251. van Os, J., and Kapur, S. (2009) Schizophrenia. *Lancet.* **374**, 635-645.

252. Barbeau, D., Liang, J.J., Robitaille, Y., Quirion, R., and Srivastava, L.K. (1995) Decreased expression of the embryonic form of the neural cell adhesion molecule in schizophrenic brains. *Proc. Natl. Acad. Sci. USA.* **92**, 2785-2789.

253. Browning, M.D., Dudek, E.M., Rapier, J.L., Leonard, S., and Freedman, R. (1993) Significant reductions in synapsin but not synaptophysin specific acitivty in the brains of some schizophrenics. *Biol. Psychiatry.* **34**, 529-535.

254. Arnold, S.E., Lee, V.M.Y., Gur, R.E., and Trojanowski, J.Q. (1991) Abnormal expression of two microtubule-associated proteins (MAP2 and MAP5) in specific subfields of the hippocampal formation in schizophrenia. *Proc. Natl. Acad. Sci. USA.* **88**, 10850-10854.

255. Weinberger, D.R., Faith Berman, K., Suddath, R., and Fuller Torrey, E. (1992) Evidence of dysfunction of a prefrontal-limbic network in schizophrenia: a magnetic resonance imaging and regional cerebral blood flow study of discordant monozygotic twins. *Am. J. Psychiatry.* **149**, 890-897.

256. Weinberger, D.R. (1993) A connectionist approach to the prefrontal cortex. *J. Neuropsych. Clin. N.* **5**, 241-253.

257. Masliah, E., Terry, R.D., Alford, M., DeTeresa, R., and Hansen, L.A. (1991) Cortical and subcortical patterns of synaptophysin-like immunoreactivity in Alzheimer's disease. *Am. J. Pathol.* **138**, 235-246.

258. Heinonen, O., Soininen, H., Sorvari, H., Kosunen, O., Paljarvi, L., Koivisto, E., and Riekkinen, P.J. (1995) Loss of synaptophysin-like immunoreactivity in the hippocampal formation is an early phenomenon in Alzheimer's disease. *Neuroscience*. **64**, 375-384.

259. Gomez Isla, T., Price, J.L., McKeel, D.W. Jr, Morris, J.C., Growdon, J.H., and Hyman, B.T. (1996) Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J. Neurosci.* **16**, 4491-4500.

260. Tiraboschi, P., Hansen, L.A., Thal, L.J., and Corey-Bloom, J. (2004) The importance of neuritic plaques and tangles to the development and evolution of AD. *Neurology*. **62**, 1984-1989.

261. Mikkonen, M., Soininen, H., Tapiola, T., Alafuzoff, I., and Miettinen, R. (1999) Hippocampal plasticity in Alzheimer's disease: changes in highly polysialylated NCAM immunoreactivity in the hippocampal formation. *Eur. J. Neurosci.* **11**, 1754-1764.

262. Geddes, J.W., Monaghan, D.T., Cotman, C.W., Lott, I.T., Kim, R.C., and Chui, H.C. (1985) Plasticity of hippocampal circuitry in Alzheimer's disease. *Science*. **230**, 1179-1181.

263. Ihara, Y. (1988) Massive somatodendritic sprouting of cortical neurons in Alzheimer's disease. *Brain Res.* **459**, 138-144.

264. Bowser, R., Kordower, J.H., and Mufson, E.J. (1997) A confocal microscopic analysis of galaninergic hyperinnervation of cholinergic basal forebrain neurons in Alzheimer's disease. *Brain Pathol.* **7**, 723-730.

265. Chang, B.S., and Lowenstein, D.H. (2003) Epilepsy. *N. Engl. J. Med.* **349**, 1257-1266.

266. Fisher, R., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., and Engel, J. (2005) Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia.* **46**, 470-472.

267. Mikkonen, M., Soininen, H., Kalviainen, R., Tapiola, T, Ylinen, A., Vapalahti, M., Paljarvi, L., and Pitkanen, A. (1998) Remodeling of neuronal circuitries in human temporal lobe epilepsy: increased expression of highly polysialylated neural cell adhesion molecule in the hippocampus and the entorhinal cortex. *Ann. Neurol.* **44**, 923-934.

268. Pekcec, A., Weinhold, B., Gerardy-Schahn, R., and Potschka, H. (2010) Polysialic acid affects pathophysiological consequences of status epilepticus. *Neuroreport.* **21**, 549-553.

269. Moberg, P.J., Pearlson, G.D., Speedie, L.J., Lipsey, J.R., Strauss, M.E., and Folstein, S.E. (1987) Olfactory recognition: differential impairments in early and late Huntington's and Alzheimer's diseases. *J. Clin. Exp. Neuropsychol.* **9**, 650-664.

270. Hamilton, J.M., Murphy, C., and Paulsen, J.S. (1999) Odor detection, learning and memory in Huntington's disease. *J. Int. Neuropsychol. Soc.* **5**, 609-615.

271. Lemiere, J., Decruyenaere, M., Evers-Kiebooms, G., Vandenbussche, E., and Dom, R. (2004) Cognitive changes in patients with Huntington's disease (HD) and asymptomatic carriers of the HD mutation—A longitudinal follow-up study. *J. Neurol.* **251**, 935-942.

272. Van der Borght, K., and Brundin, P. (2007) Reduced expression of PSA-NCAM in the hippocampus and piriform cortex of the R6/1 and R6/2 mouse models of Huntington's disease. *Exp. Neurol.* **204**, 473-478.

273. Murphy, K.J., O'Connell, A.W., and Regan, C.M. (1996) Repetitive and transient increases in hippocampal neural cell adhesion molecule polysialylation state following multitrial spatial training. *J. Neurochem.* **67**, 1268-1274.

274. Van der Borght, K., Wallinga, A.E., Luiten, P.G., Eggen, B.J., and Van der Zee, E.A. (2005) Morris water maze learning in two rat strains increases the expression of the polysialylated form of the neural cell adhesion molecule in the dentate gyrus but has no effect on hippocampal neurogenesis. *Behav. Neurosci.* **119**, 926-932.

275. Dall'Olio, F. and Chiricolo, M. (2001) Sialyltransferases in cancer. *Glycoconj. J.* **18**, 841-850.

276. Petridis, A.K., Wedderhopp, H., Hugo, H.H., and Mehdorn, H.M. (2009) Polysialic acid overexpression in malignant astrocytomas. *Acta. Neurochir.* **151**, 601-604.

277. Suzuki, Ma., Suzuki, Mi., Nakayama, J., Suzuki, A., Angata, K., Chen, S., Sakai, K., Hagihara, K., Yamaguchi, Y., and Fukuda, M. (2005) Polysialic acid facilitates tumor invasion by glioma cells. *Glycobiology.* **15**, 887-894.

278. Hildebrandt, H., Becker, C., Gluer, S., Rosner, H., Gerardy-Schahn, R., and Rahmann, H. (1998). Polysialic acid on the neural cell adhesion molecule correlates with expression of polysialyltransferases and promotes neuroblastoma cell growth. *Cancer Res.* **58**, 779-784.

279. Komminoth, P., Roth, J., Saremaslani, P., Matias-Guiu, X., Wolfe, H.J., and Heitz, P.U. (1994) Polysialic acid of the neural cell adhesion molecule in the human thyroid: a marker for medullary thyroid carcinoma and primary C-cell hyperplasia. An immunohistochemical study on 79 thyroid lesions. *Am. J. Surg. Pathol.* **18**, 399-411.

280. Lantuejoul, S., Moro, D., Michalides, R.J., Brambilla, C., and Brambilla, E. (1998) Neural cell adhesion molecules (NCAM) and NCAM-PSA expression in neuroendocrine lung tumors. *Am. J. Surg. Pathol.* **22**, 1267-1276.

281. Buckner, J.C., Brown, P.D., O'Neill, B.P., Meyer, F.B., Wetmore, C.J., and Uhm, J.H. (2007) Central nervous system tumors. *Mayo Clin. Proc.* **82**, 1271-1286.

282. Miyahara, R., Tanaka, F., Nakagawa, T., Matsuoka, K., Ish, K., and Wada, H. (2001) Expression of Neural Cell Adhesion Molecules (Polysialylated Form of Neural Cell Adhesion Molecule and L1-Cell Adhesion Molecule) on Resected Small Cell Lung Cancer Specimens: In Relation to Proliferation State. *J. Surg. Oncol.* **77**, 49-54.

283. Glüer, S., Schelp, C., von Schweinitz, D., and Gerardy-Schahn, R. (1998) Polysialylated neural cell adhesion molecule in childhood rhabdomyosarcoma. *Pediatr. Res.* **43**, 145-147.

284. Seidenfaden, R., Gerardy-Schahn, R., and Hildebrandt, H. (2000) Control of NCAM polysialylation by the differential expression of polysialyltransferases ST8SiaII and ST8SiaIV. *Eur. J. Cell Biol.* **79**, 680-688.

285. Roesler, J., Srivatsan, E., Moatamed, F., Peters, J., and Livingston, E.H. (1997) Tumor suppressor activity of neural cell adhesion molecule in colon carcinoma. *Am. J. Surg.* **174**, 251-257.

286. Kameda, K., Shimada, H., Ishikawa, T., Takimoto, A., Momiyama, N., Hasegawa, S., Misuta, K., Nakano, A., Nagashima, Y., and Ichikawa, Y. (1999) Expression of highly polysialylated neural cell adhesion molecule in pancreatic cancer neural invasive lesion. *Cancer Lett.* **137**, 201-207.

287. Tezel, E., Kawase, Y., Takeda, S., Oshima, K., and Nakao, A. (2001) Expression of neural cell adhesion molecule in pancreatic cancer. *Pancreas.* **22**, 122-125.

288. Thomas, L. A., Akins, M. R., and Biederer, T. (2008) Expression and adhesion profiles of SynCAM molecules indicate distinct neuronal functions. J. Comp. Neurol. **510**, 47-67.

289. Galuska, S. P., Rollenhagen, M., Kaup, M., Eggers, K., Oltmann-Norden, I., Schiff, M., Hartmann, M., Weinhold, B., Hildebrandt, H., Geyer, R., Mühlenhoff, M., and Geyer, H. (2010) Synaptic cell adhesion molecule 1 SynCAM 1 is a target for polysialylation in postnatal mouse brain. *Proc. Natl. Acad. Sci.* **107**, 10250-10255.

290. Levinson, J.N., Chery, N., Huang, K., Wong, T.P., Gerrow, K., Kang, R., Prange, O., Wang, Y.T., and El-Husseini, A. (2005) Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1 beta in neuroligin-induced synaptic specificity. *J Biol Chem* **280**, 17312–17319.

291. Pellet-Many, C., Frankel, P., Jia, H., and Zachary, I. (2008) Neuropilins: structure, function, and role in disease. *Biochem. J.* **411**, 211-226.

292. Bagri, A., Tessier-Lavigne, M., and Watts, R.J. (2009) Neuropilins in tumor biology. *Clin. Cancer Res.* **15**, 1860-1864.

293. Curreli, S., Arany, Z., Gerardy-Schahn, R., Mann, D., and Stamatos, N. M. (2007) Polysialylated neuropilin-2 is expressed on the surface of human dendritic cells and modulates dendritic cell-T lymphocyte interactions. *J. Biol. Chem.* **282**, 30346-30356.

294. Rey-Gallardo, A., Escribano, C., Delgado-Martin, C., Rodriguez-Fernandez, J. L., Gerardy-Schahn, R., Rutishauser, U., Corbi, A. L., and Vega, M. A. (2010) Polysialylated neuropilin-2 enhances human dendritic cell migration through the basic C-terminal region of CCL21. *Glycobiology*. **20**, 1139-1146.

295. Gray, M.J., Van Buren, G., Dallas, N.A., Xia, L., Wang, X., Yang, A.D., Somcio, R.J., Lin, Y.G., Lim, S., Fan, F., Mangala, L.S., Arumugam, T., Logsdon, C.D., Lopez-Berestein, G., Sood, A.K., and Ellis, L.M. (2008) Therapeutic targeting of neuropilin-2 on colorectal carcinoma cells implanted in the murine liver. *J. Natl. Cancer Inst.* **100**, 446.

296. Bielenberg, D.R., and Klagsbrun, M. (2007) Targeting endothelial and tumor cells with semaphorins. *Cancer Metastasis Rev.* **26**, 421-431.

297. Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M.-A., Samyn-Petit, B., Julien, S., and Delannoy, P. (2001) The human sialyltransferase family. *Biochimie.* **83**, 727-737.

298. Grabenhorst, E. and Conradt, H.S. (1999) The cytoplasmic, transmembrane, and stem regions of glycosyltransferases specify their *in vivo* functional sublocalization and stability in the Golgi. *J. Biol. Chem.* **274**, 36107-36116.

299. Datta, A.K. and Paulson, J.C. (1994) The sialyltransferase "sialylmotif" participates in binding the donor substrate CMP-NeuAc. *J. Biol. Chem.* **270**, 1497-1500.

300. Datta, A.K., Sinha, A., and Paulson, J.C. (1998) Mutation of the sialyltransferase S-sialylmotif alters the kinetics of the donor and acceptor substrates. *J. Biol. Chem.* **273**, 9608-9614.

301. Geremia, R.A., Harduin-Lepers, A., and Delannoy, P. (1997) Identification of two novel conserved amino acid residues in eukaryotic sialyltransferases: implications for their mechanism of action. *Glycobiology.* **7**, v-vii.

302. Kitazume-Kawaguchi, S., Kabata, S., and Arita, M. (2001) Differential biosynthesis of polysialic or disialic acid structure by ST8Sia II and ST8Sia IV. *J. Biol. Chem.* **276**, 15696-15703.

303. Kapitonov, D. and Yu, R.K. (1999) Conserved domains of glycosyltransferases. *Glycobiology.* **9**, 961-978.

304. Jeanneau, C., Chazalet, V., Auge, C., Soumpasis, D.M., Harduin-Lepers, A., Delannoy, P., Imberty, A., and Breton, C. (2004) Structure-function analysis of the human sialyltransferase ST3Gal I: role of *N*-glycosylation and a novel conserved sialylmotif. *J. Biol. Chem.* **279**, 13461-13468.

305. Foley, D.A., Swartzentruber, K.G., and Colley, K.J. (2009) Identification of sequences in the polysialyltransferases ST8Sia II and ST8Sia IV that are required for the protein-specific polysialylation of the neural cell adhesion molecule, NCAM. *J. Biol. Chem.* **284**, 15505-15516.

306. Nakata, D., Zhang, L., and Troy, F.A. (2006) Molecular basis for polysialylation: A novel polybasic polysialyltransferase domain (PSTD) of 32 amino acids unique to the α2, 8-polysialyltransferases is essential for polysialylation. *Glycoconj. J.* **23**, 423-436.

307. Angata, K., Yen, T.-Y., El-Battari, A., Macher, B.A., and Fukuda, M. (2001) Unique Disulfide Bond Structures Found in ST8Sia IV Polysialyltransferase Are Required for Its Activity. *J. Biol. Chem.* **276**, 15369-15377.

308. Close, B.E., Wilkinson, J.M., Bohrer, T.J., Goodwin, C.P., Broom, L.J., and Colley, K.J. (2001) The polysialyltransferase ST8Sia II/STX: posttranslational processing and the role of autopolysialylation in the polysialylation of neural cell adhesion molecule. *Glycobiology.* **11**, 997-1008.

309. Mühlenhoff, M., Eckhardt, M., Bethe, A., Frosch, M., and Gerardy-Schahn, R. (1996) Autocatalytic polysialylation of polysialyltransferase-1. *EMBO J.* **15**, 6943-6950.

310. Close, B.E., Tao, K., and Colley, K.J. (2000) Polysialyltransferase-1 autopolysialylation is not requisite for polysialylation of neural cell adhesion molecule. *J. Biol. Chem.* **275**, 4484-4491.

311. Close, B.E., Mendiratta, S.S., Geiger, K.M., Broom, L.J., Ho, L-L., and Colley, K.J. (2003) The minimal structural domains required for neural cell adhesion molecule polysialylation by PST/ST8Sia IV and STX/ST8Sia II. *J. Biol.Chem.* **278**, 30796-30805.

312. Mendiratta, S.S., Sekulic, N., Lavie, A., and Colley, K.J. (2005) Specific amino acids in the first fibronectin type III repeat of the neural cell adhesion molecule play a role in its recognition and polysialylation by the polysialyltransferase ST8Sia IV/PST. *J. Biol. Chem.* **280**, 32340-32348.

313. Nelson, R.W., Bates, P.A., and Rutishauser, U. (1995) Protein determinants for specific polysialylation of the neural cell adhesion molecule. *J. Biol. Chem.* **270**, 17171-17179.

314. Thompson, M.G., Foley, D.A., Swartzentruber, K.G., and Colley, K.J. (2011) Sequences at the interface of the fifth immunoglobulin domain and first fibronectin type III repeat of the neural cell adhesion molecule are critical for its polysialylation. *J. Biol. Chem.* **286**, 4525-4534.

315. Mendiratta, S.S., Sekulic, N., Hernandez-Guzman, F.G., Close, B.E., Lavie, A., and Colley, K.J. (2006) A novel α-Helix in the first fibronectin type III repeat of the neural cell adhesion molecule is critical for *N*-glycan polysialylation. *J. Biol. Chem.* **281**, 36052-36059.

316. Thompson, M.G., Foley, D.A., and Colley, K.J. (2013) The poylsialyltransferases interact with sequences in two domains of the neural cell adhesion molecule to allow its polysialylation. *J. Biol. Chem.* **288**, 7282-7293.

317. Foley, D.A., Swartzentruber, K.G., Lavie, A., and Colley, K.J. (2010) Structure and mutagenesis of neural cell adhesion molecule domains: evidence for the flexibility in the placement of polysialic acid attachment sites. *J. Biol. Chem.* **285**, 27360-27371.

318. Foley, D.A., Swartzentruber, K.G., Thompson, M.G., Mendiratta, S.S., and Colley, K.J. (2010) Sequences from the first fibronectin type III repeat of the neural cell adhesion molecule allow O-glycan polysialylation of an adhesion molecule chimera. *J. Biol. Chem.* **285**, 35056-35067.

319. Yoshihara, Y., Kawasaki, M., Tamada, A., Fujita, H., Hayashi, H., Kagamiyama, H., and Mori, K. (1997) OCAM: A new member of the neural cell adhesion molecule family related to zone-to-zone projection of olfactory and vomeronasal axons. *J. Neurosci.* **17**, 5830-5842.

320. Alenius, M., and Bohm, S. (1997) Identification of a novel neural cell adhesion molecule-related gene with a potential role in selective axonal projection. *J. Biol. Chem.* **272**, 26083-26086.

321. Angata, K., Chan, D., Thibault, J., and Fukuda, M. (2004) Molecular dissection of the ST8Sia IV polysialyltransferase. *J. Biol. Chem.* **279**, 25883-25890.

322. Angata, K., Suzuki, M., McAuliffe, J., Ding, Y., Hindsgaul, O., and Fukuda, M. (2000) Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct α2,8-Sialyltransferases, ST8Sia IV (PST), ST8Sia II (STX), and ST8Sia III. *J. Biol. Chem.* **275**, 18594-18601.

323. Heimburg-Molinaro, J., Lum, M., Vijay, G., Jain, M., Almogren, A., and Rittenhouse-Olson, K. (2011) Cancer vaccines and carbohydrate epitopes. *Vaccine.* **29**, 8802-8826.

324. Amoureux, M.C., Coulibaly, B., Chinot, O., Loundou, A., Metellus, P., Rougon, G., and Figarella-Branger, D. (2010) Polysialic acid neural cell adhesion molecule (PSA-NCAM) is an adverse prognosis factor in glioblastoma, and regulates olig2 expression in glioma cell lines. *BMC Cancer.* **10**, 91.

325. Krug, L.M., Ragupathi, G., Ng, K.K., Hood, C., Jennings, H.J., Guo, Z., Kris, M.G., Miller, V., Pizzo, B., Tyson, L., Baez, V., and Livingston, P.O. (2004) Vaccination of small cell lung cancer patients with polysialic acid or N-propionylated polysialic acid conjugated to keyhole. *Clin. Cancer Res.* **10**, 916-23.

326. Stanley, P. (1984) Glycosylation mutants of animal cells. *Annu. Rev. Genet.* **18**, 525-552.

327. Jones, D.T. (1999) Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol.* **292**, 195-202.

328. Moreira, I.S., Fernandes, P.A., and Ramos, M.J. (2007) Hot spots. A review of the protein-protein interface determinant amino acid residues. *Proteins.* **68**, 803-812.

329. Bogan, A.A., and Thorn, K.S. (1998) Anatomy of hot spots in protein interfaces. *J. Mol. Biol.* **280**, 1-9.

330. Laha, K.T., and Wagner, D.A. (2011) A state-dependent salt-bridge interaction exists across the β/α intersubunit interface of the GABA A receptor. *Mol. Pharmacol.* **79**, 662-671.

331. Mühlenhoff, M., Rollenhagen, M., Werneburg, S., Gerardy-Schahn, R., and Hildebrandt, H. (2013) Polysialic acid: versatile modification of NCAM, SynCAM 1 and neuropilin-2. *Neurochem. Res.* In Press.

332. Angata, K., Long, J.M., Bukalo, O., Lee, W., Dityatev, A., Wynshaw-Boris, A., Schachner, M., Fukuda, M., and Marth, J.D. (2004) Sialyltransferase ST8Sia-II assembles a subset of polysialic acid that directs hippocampal axonal targeting and promotes fear behavior. *J. Biol. Chem.* **279**, 32603-32613.

333. Tanaka, F., Otake, Y., Nakagawa, T., Kawano, Y., Miyahara, R., Li, M., Yanagihara, J., Fujimoto, I., Ikenaka, K., and Wada, H. (2000) Prognostic significance of polysialic acid expression in resected non-small cell lung cancer. *Cancer Res.* **60**, 3072-3080.

334. Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, ,R., McGettigan, P.A., McWilliam H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*. **23**, 2947-2948.

335. Ye, L., Wu, Z., Eleftheriou, M., and Zhoi, R. (2007) Single-mutation-induced stability loss in protein lysozyme. *Biochem. Soc. T.* **35**, 1551-1557.

336. Opat, A.S., Houghton, F., and Gleeson, P.A. (2000) *Medial* Golgi but not *Late* Golgi glycosyltransferases exist as high molecular weight complexes: role of lumenal domain in complex formation and localization. *J. Biol. Chem.* **275**, 11836-11845.

337. Yamaguchi, N. and Fukuda, M.N. (1995) Golgi retention mechanism of β-1,4-Galactosyltransferase: membrane-spanning domain-dependent homodimerization and association with α- and β-tubulins. *J. Biol. Chem.* **270**, 12170-12176.

338. El-Battari, A., Prorok, M., Angata, K., Mathieu, S., Zerfaoui, M., Ong, E., Suzuki, M., Lombardo, D., and Fukuda, M. (2003) Different glycosyltransferases are differentially processed for secretion, dimerization, and autoglycosylation. *Glycobiology.* **13**, 941-853.

339. Ma, J. and Colley, K.J. (1996) A disulfide-bonded dimer of the Golgi β-Galactoside α2,6-Sialyltransferase is catalytically inactive yet still retains the ability to bind galactose. **J. Biol. Chem. 271**, 7758-7766.

340. Qian, R., Chen, C., and Colley, K.J. (2001) Location and mechanism of α2,6-Sialyltransferase dimer formation: role of cysteine residues in enzyme dimerization, localization, activity, and processing. *J. Biol. Chem.* **276**, 28641-28649.

341. Fogel, A.I., Li, Y., Giza, J., Wang, Q., Lam, T.T., Modis, Y., and Biederer, T. (2010) N-glycosylation at the SynCAM (Synaptic Cell Adhesion Molecule) immunoglobulin interface modulates synaptic adhesion. *J. Biol. Chem.* **285**, 34864-34874.

342. Rollenhagen, M., Kuckuck, S., Ulm, C., Hartmann, M., Galuska, S.P., Geyer, R., Geyer, H., and Mühlenhoff, M. (2012) Polysialylation of the synaptic cell adhesion molecule 1 (SynCAM 1) depends exclusively on the polysialyltransferase ST8SiaII *in vivo*. *J. Biol. Chem.* **287**, 35170-35180.

343. Aricescu, A.R., Hon, W.C., Siebold, C., Lu, W., van der Merwe, P.A., and Jones, E.Y. (2006) Molecular analysis of receptor protein tyrosine phosphatase mu-mediated cell adhesion. *EMBO J.* **25**, 701-712.

344. Rao, F.V., Rich, J.R., Raki, B., Buddai, S., Schwartz, M.F., Johnson, K., Bowe, C., Wakarchuk, W.W., Defrees, S., Withers, S.G., and Strynadka, N.C. (2009) Structural insights into mammalian sialyltransferases. *Nat. Struct. Mol. Biol.* **16**, 1186-1188.

345. Chiu, C.P., Watts, A.G., Lairson, L.L., Gilbert, M., Lim, D., Wakarchuk, W.W., Withers, S.G., and Strynadka, N.C. (2004) Structural analysis of the sialyltransferase CstII from *Campylobacter jejuni* in complex with a substrate analog. *Nat. Struct. Mol. Biol.* **11**, 163-170.

346. Kim, D.U., Yoo, J.H., Lee, Y.J., Kim, K.S., and Cho, H.S. (2008) Structural analysis of sialyltransferase Pm0188 from *Pasturella multocida* complexed with donor analogue and acceptor sugar. *BMB Rep.* **41**, 48-54.

APPENDIX

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