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Improved methods to characterize the length and quantity of highly unstable PolySialic acids subject category: (Carbohydrates, chromatographic techniques)

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ABSTRACT

Polysialic acid (polySia) is a linear homopolymer of α2-8-linked sialic acids that is highly expressed during early stages of mammalian brain development and modulates a multitude of cellular functions. While degree of polymerization (DP) can affect such functions, currently available methods do not accurately characterize this parameter, because of the instability of the polymer. We developed two improved methods to characterize the DP and total polySia content in biological samples. PolySia chains with exposed reducing termini can be derivatized with DMB for subsequent HPLC analysis. However, application to biological samples of polySia-glycoproteins requires release of polySia chains from the underlying glycan, which is difficult to achieve without concurrent partial hydrolysis of the α2-8-linkages of the polySia chain, affecting its accurate characterization. We report an approach to protect internal α2-8sia linkages of long polySia chains, using previously known esterification conditions that generate stable polylactone structures. Such polylactonized molecules are more stable during acid hydrolysis release and acidic DMB derivatization. Additionally, we used the highly specific Endoneuraminidase-NF enzyme to discriminate polysialic acid and other sialic acid and developed an approach to precisely measure the total content of polySia in a biological sample. These two methods provide improved quantification and characterization of polySia.

1. Introduction

Polysialic acid (polySia) is a linear homopolymer of sialic acids (Sias) in α2-8 linkages. PolySia was first discovered in the polysaccharide capsule of *E. Coli* $K_{235}L + O$ in 1957 [1]. Some four years after discovering sialic acids in bacteria, Guy Barry called the capsular polysaccharide of this strain of *E. Coli* "colominic acid," and reported that the structure of this molecule consists of a polymer of repeating residues of *N*-acetylneuraminic acid (Neu5Ac). Barry suggested that, given the ongoing explosion of discoveries related to sialic acids in the 1950s, such a polymeric structure of sialic acids may have some relevance in mammals [2]. In fact, this prediction proved to be correct [3–7], and a common antigen found in vertebrate brains would later be identified as a similar polymer of sialic acids extended upon the N-glycans of the neural cell adhesion molecule (NCAM) [8–10]. Notably, *E. coli* K1 expressing PolySia is a common cause of neonatal and pediatric meningitis [11–13], and can also cause infections in adults [14,15].

In vertebrates, polySia is highly expressed during embryonic brain development and, in mice, reaches peak expression levels perinatally [16]. During postnatal mouse brain development, the amounts of polySia remain high during the first week, before rapidly declining between postnatal days 9–17. This decrease continues into adulthood [17–19]. In both adult rodents and humans, polySia is selectively expressed in areas where neurogenesis persists [20–22]. Functionally, polySia forms a highly hydrated structure on the cell surface with a steric effect ("repulsive field") that directly affects cell-cell contacts and interactions of cell surface receptors and matrix components, while also sequestering and concentrating soluble ligands like FGF2 and BDNF ("attractive field"), enabling critical modulatory roles in many neural processes [23]. PolySia-N-glycan structures depend on expression and activity of two enzymes ST8Sia-II and ST8Sia-IV, which act either independently or cooperatively [24–27].

Accurately determining DP of polySia in biological samples has remained a technical challenge. HPLC-based analysis has been used in

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the characterization of polySia to determine the structural characteristics, primarily the degree of polymerization (DP) [26,28]. Another approach to interpret the chain length of polySia include analysis of the relative abundance of the terminal sialic acids compared with the internal sialic acids. This can be accomplished either by comparing the relative reactivity of antibodies that recognize the polySia chain (mAb 10E3) to antibodies that bind along the polymer in an abundance relative to the length (mAb 735) [29]. Alternatively, a chemical modification can be used to demarcate the non-reducing terminal sialic acid by mild periodate oxidation which cleaves a 2-carbon glycol from the side-chain of exposed sialic acids leaving a 7-carbon sugar in the non-reducing terminal position, while internal residues remain protected from reduction in a 9-carbon form [28]. The C7 and C9 sugars can then be released to monomers and the relative abundance quantitated by C18-HPLC. However, even a small amount of contaminating monosialyl residues can confound the accuracy of this method.

Fluorometric analysis of sialic acids is a useful tool for quantitatively studying relative abundance of sialic acid forms, enabling highly sensitive detection of biological samples which typically contain sialic acids on the order of picomoles to femtomoles. 1,2-diamino-4,5-methylenedioxygenzene (DMB) selectively labels alpha-keto acids like sialic acids, producing a covalent derivative that can be separated by HPLC with a quantitative fluorescence readout. Derivatization of sialic acid was first accomplished by using acidic conditions to protonate the sialic acid enabling a reaction aldehyde side chain of the fluor DMB via Schiff-base mechanism at 50 °C. The acid used was 0.7 M HCl [30], however, because sialic acid and its derivatives are labile this was later optimized to use milder conditions using 1 M acetic acid (AcOH) [28]. Later application of DMB derivatization to polysialic acid was accomplished by dropping the temperature of the reaction to 4 °C or 10 °C and using a smaller quantity of the stronger triflouroacetic acid (TFA) at 20 mM [28].

Applications to biological samples of sialylated-glycoproteins first require release of sialic acid from the underlying glycan structure, which in mammals is an α2-3 or α2-6 linkage to galactose. This release can be efficiently accomplished enzymatically by a sialidase, or chemically by a simple incubation in a mild or strong acid at high temperature [31]. But this step poses a significant challenge to the analysis of polySia chains in biological samples: exosialidases can no longer work on this internal bond, and the instability of the internal α2-8-glycosidic bonds of polySia can undergo spontaneous hydrolysis via intramolecular cleavage in a temperature and pH dependent manner [32], resulting in degradation of polySia prior to analysis, thus affecting its detectable DP [33]. Standard analysis using acid hydrolysis release and DMB derivatization under acidic conditions typically yields polySia fragments of DP ~30–50 from biological samples. However, DP > 400 in embryonic brain has been reported following release using endo-β-galactosidase, an enzyme that selectively releases the rare polySia chains linked via a poly-N-acetyllactosamine motif [34]. The release of all polySia chains, rather than an enzymatically selected subset, from the underlying glycoprotein remains a challenge for the field. ELISA-capture has also been used as a method to identify polysialic carriers in serum, where a number of non-NCAM polysialylated proteins exist [35]. A recent comprehensive review of methods for polySia analysis summarized these difficulties, concluding that "a methodology for truly accurate determination of polySia chain length remains elusive" [33].

Classic studies in polymer chemistry showed that the resonant properties of poly-acids determined that the pK_a of carboxyl groups increases proportionally with the DP of the molecule [36]. This has consequences on the labile α2-8 glycosidic linkages of polysialic acid, as they experience a self-catalyzed intramolecular cleavage [32], dependent upon the protonation and thus the pK_a of the neighboring carboxylate groups. As the polymer is extended and carboxylate groups become protonated, the internal most bonds will become increasingly susceptible to this catalyzed hydrolysis [32]. Because of this protonation, when the very long polySia chains found in mammalian tissues are subjected to standard sialic acid DMB derivatization conditions of pH 3–5, internal linkages experience catalyzed hydrolysis even at 10 °C, or 4 °C.

Previous methods involving DMB analysis of polysialylated glycoconjugates took advantage of the low-rate of unpredictable hydrolysis by directly subjecting polysialylated structures to DMB derivatization conditions. Random hydrolysis of internal bonds produces fragments of DP 20–100 with reducing sialic acid residues exposed for the DMB labeling chemistry to proceed [37]. However, this unpredictable rate of hydrolysis also presents challenges to accurately determine the DP. One solution is minimize hydrolysis is via lactonization. We achieve lactonization-protection of the α2-8 glycosidic linkages using ice cold-acidic conditions. Under these conditions, the C9 hydroxyl group of each sialic acid undergoes esterification with the carboxylate of the adjacent sialic acid to form a stable6-carbon lactone structure (Fig. 1A) [38–40]. Due to the spatial arrangement between galactose and a 2–3 linked sialic acid a similar lactone ring can be formed by using a highly reactive catalyst [41], however, it is unlikely that such a reaction could proceed under milder or biologically relevant conditions. An early study of the lactonization properties of polysialic acid suggested that lactonization may offer protection during acid hydrolysis [39]. This study showed protection of the highly abundant oligo/poly-Neu5Gc (DP ≤ 11) structures from salmon eggs prior to PAD (pulse amperometric detection). Notably, lactonization was used to enable MALDI-TOF-Mass Spectrometry of polysialic acid chains [42]. Here, we apply the lactonization-protection in mammalian glycoproteins containing large poly-Neu5Ac structures, prior to DMB labeling, and fluorescence detection and took advantage of the polysialic acid specificity of Endo-N enzyme for total polysialic acid quantification.

2. Materials and methods

2.1. Lactonization and mild acid hydrolysis of colominic acid

10 nmol (as Neu5Ac) of Colominic acid in 50 mM Tris-HCl pH 8.0 was lactonized by incubation overnight in an ice water bath after the addition of ice-cold HCl to final concentration of 1 M induce lactonization, or water as a non-lactonized control. After overnight lactonization, samples were frozen at −80 °C and lyophilized to remove HCl. Dried samples of colominic acid were then resuspended in 50 μl 2 M AcOH and subjected to a 90 min incubation at 80 °C, to mimic the conditions known to release terminal Sias from biological glycoconjugates.

2.2. HPLC methods

After derivatization, samples were immediately prepared for HPLC analysis. Samples were centrifuged at maximum speed for 20 min to prevent injecting any non-soluble particulate into the HPLC system. After centrifugation, samples were transferred into auto-sampler vials for injection. Volumes between 20 and 80 μl were injected, depending on concentration of samples.

HPLC chromatography was run on a Hitachi LaChrom Elite HPLC system fitted with a CARBOPAC PA200 Analytical Column (3 × 250 mm, Thermo scientific P/N 062896), and CARBOPAC PA200 Guard Column (3 × 50 mm, Thermo scientific P/N 062895). Fluorescence was measured at 373 nm excitation and 448 nm emission (Jasco detector). Anion exchange was accomplished using a flow of 0.5 ml/min and the following gradient of Milli-Q water (E1) and 1 M sodium nitrate (E2): 0 min = 0% E2, 2 min = 2% E2, 9 min = 10% E2, 39 min = 16% E2, 99 min = 31% E2, 100 min = 2% E2, 110 min = 2% E2.

Chromatograms were produced and analyzed with OpenLab Chromatography Data System (CDS) EZChrom Edition (Agilent Technologies).

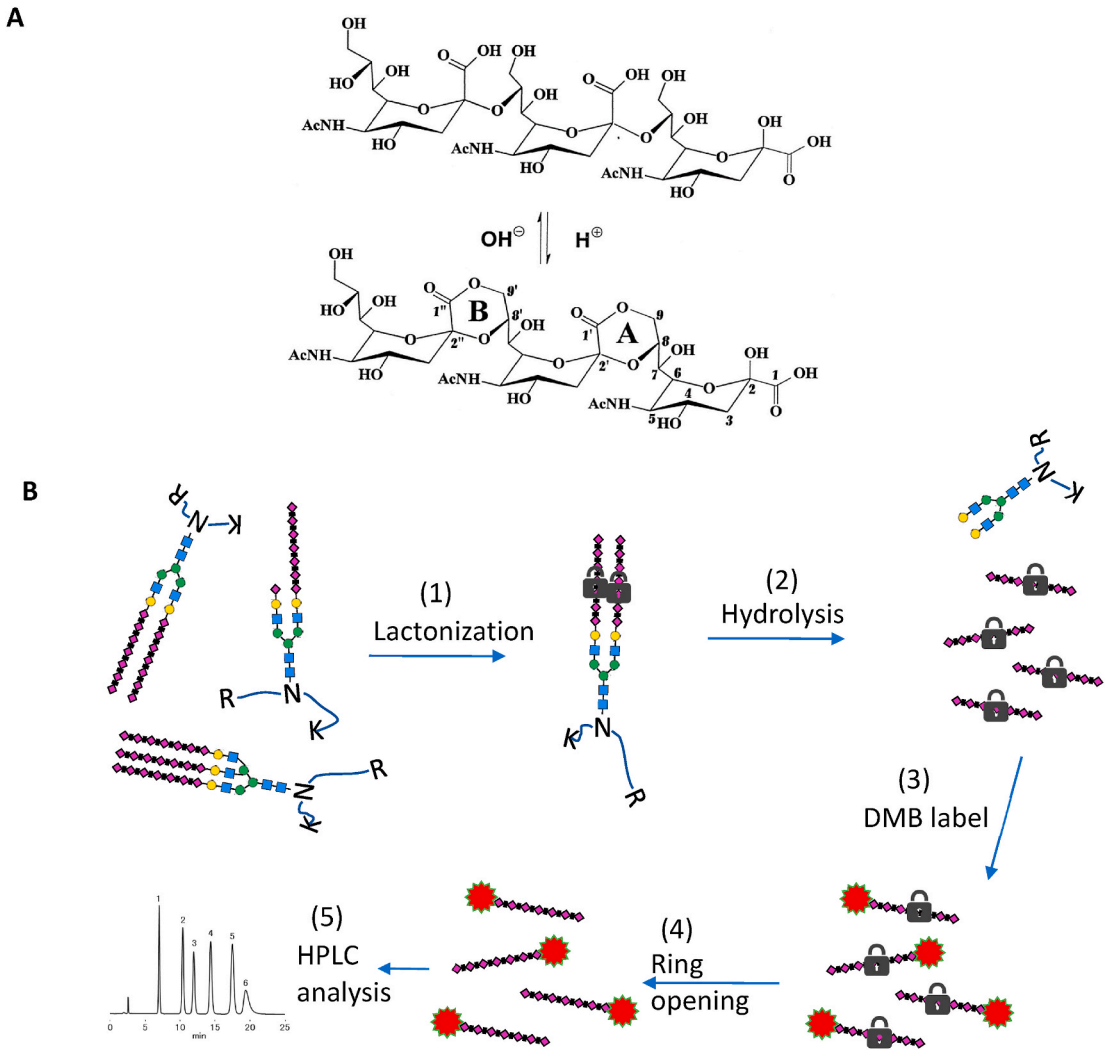

Fig. 1. Overview of polysialic acid lactonization, and Lactonization-Protection-DMB procedure **A)** Structure of α2-8 linked Neu5Ac (above), and the same following acidic lactonization. **B)** Overview of Lactonization-Protection-DMB procedure for DP analysis of glycoprotein polysialic acid moieties: (1) polysialic acid moeties are lactonized, (2) mild acid hydrolysis releases sialic acid structures from underlying glycans, (3) sialic acid structures are labeled with DMB fluorophore, (4) lactonization is reversed, (5) labeled structures are quantitated via anion exchange HPLC.

2.3. Brain tissue homogenization and delipidation

Mice were euthanized by isofluorane inhalation followed by decapitation, and the whole brain was collected immediately after dissection. Brain mass was recorded, and tissue was placed on ice in a glass centrifuge tube with ice cold water at a 4:1 w/w ratio of water:brain tissue. Keeping tube on ice, tissue was thoroughly homogenized, for about 1 min, on a polytron at high speed. 1:1 chloroform:methanol mixture was added at 20X volume of water/brain homogenate and mixed vigorously by polytron, followed by centrifugation at 1200×*g* for 15 min at 4 °C. Supernatant containing lipids and low molecular weight molecules was carefully removed and discarded, leaving a pellet containing brain glycoproteins and other macromolecules. To wash pellet, fresh 10:10:1 chloroform:methanol:water mixture was added at the previous volume before polytron mixing and centrifugation as before.

After final wash, samples were left on ice. After excess organic solvent was completely evaporated 50 mM Tris-HCl pH 8.0 was added at 2x original brain mass. Pellet was suspended completely with repeated pipetting.

2.4. Proteinase digestion

Proteinase K (Invitrogen #25530031) was used to digest the protein component of brain glycoprotein samples, producing glyopeptides ready for analysis. Glycoproteins suspended in 50 mM Tris-HCl pH 8.0 were treated with 200 ng/μl proteinase K (0.01 vol 20 mg/ml stock enzyme). Samples were then incubated overnight at 37 °C with rapid mixing. 1 mM phenylmethylsulfonyl flouride (PMSF) was then added to inhibit proteinase (0.01 vol 100 mM stock PMSF in isopropanol). Samples were then washed using a 3 K-cutoff centrifugal filter unit (Sigma-Millipore #UFC9003), with 3 serial dilution-concentration steps: dilution in 15 ml 50 mM Tris-HCl pH 8.0 followed by concentration.

2.5. Lactonization and mild acid hydrolysis of mouse brain glycopeptides

100 μl of brain glycopeptides (derived from ~50 mg brain tissue) was placed in an ice water bath, and 100 μl of ice cold 2 M HCl (or H2O for the non-lactonized control) was quickly added and mixed (final concentration 1 M HCl). The samples were incubated overnight in ice water to induce lactonization. The samples were then centrifuged at maximum speed (17000 g) at 4 °C for 30 min, frozen at −80 °C, and lyophilized until dry. The dried lactonized glycopeptides were then

suspended in 100 μl 2 M AcOH and heated at 80 °C to hydrolyze any non-lactonized sialic acid linkages, including the internal 2–3 and 2–6 linkages connecting the polysialic acid chains to their N-glycan antennae.

2.6. Enzymatic digestion of polysialic acid and quantitation of endoN-sensitive Neu5Ac

50 μl of brain glycopeptides prepared (originating from 25 mg brain tissue) was treated with 1 μl endoneuraminidase-NF on ice for 90 mintues (2 mg/ml enzyme stock, enzyme was a kind gift from Rita Gerardy-Schahn). Samples were then filtered through 3 k centricon filter, and flow-through fractions were collected for DMB labeling. After DMB analysis, oligosialic acid products of endoN were identified and peak areas calculated using OpenLab Chromatography Data System (CDS) EZChrom Edition (Agilent Technologies). For each sample total relative endoN-sensitive Neu5Ac was determined by calculating the sum of product oligomers, with each oligomer peak area multiplied by its respective DP. 1.25 μg of brain tissue were used for each injection, and 25 pmol of colominic acid was analyzed in parallel and used as a standard.

2.7. DMB labeling

DMB labeling was accomplished in a reaction containing 1.35 M DMB, 1 M acetic acid, 9 mM sodium hydrosulfite, and 0.5 M β-mercaptoethanol. Samples containing sialic acid and 2 M acetic acid were placed on ice, and 50 μl of a 2X mix containing the remaining reaction ingredients (DMB, $NaSO_2$, and β-ME) was added. This reaction was incubated away from light, at 4 °C, with end-over-end mixing or at 50 °C for 2 h. The 4 °C samples were labeled for 40 h. After DMB labeling, 14 μl 10 N NaOH was added, and samples were mixed thoroughly with vortexing. NaOH neutralizes acetic acid, reverses esterification of the polylactone structure, and frees carboxylate groups to restore negative charge for anion exchange separation.

3. Results and discussion

3.1. Overview of methods

To more accurately determine the DP in biological examples, we developed an approach to protect internal

α2-8sia linkages of long polySia chains during acid hydrolysis and subsequent DMB derivatization. We achieve this by first inducing the formation of stable lactone-ring structures (Fig. 1A and B – step 1) along the length of the polySia, prior to proceeding with acid hydrolysis (Fig. 1B – step 2) and DMB labeling (Fig. 1B – step 3). This lactonized molecule remains stable under acid hydrolysis release and during acidic DMB derivatization. Subsequent addition of base reverses lactonization (Fig. 1B – step 4) before separation of the labeled polySias with anion exchange HPLC (Fig. 1B – step 5) and analysis using fluorescence detection of DMB labels. Free polymers of sialic acid, such as bacterial colominic acid which is collected from *E. Coli* culture and is not covalently linked at the reducing end, can be readily labeled by DMB. However, accomplishing release from the underlying glycan structure (Fig. 1B – step 2) has remained an obstacle for the analysis of mammalian polysialylated glycan structures. This lactonization-DMB allows for improved characterization of the degrees of polymerization, but it does not provide quantitation of polySia content.

To quantitate polySia, we take advantage of the highly specific endosialidase Endoneuraminidase-NF (EndoN), to quantitate the total amount of polySia in a sample. EndoN is an endosialidase from the bacteriophage that evolved to specifically targets the polySia capsular polysaccharide of *E. Coli* K1. EndoN cleaves α2-8Sia linkages within a chain of sias with degree of polymerization of 5 or greater [43]. We applied the specificity of the EndoN enzyme as a tool to selectively release all polySia from brain samples that we also analyzed by lactonization-DMB to derive a parallel absolute quantitation of polySia content.

3.2. Lactonization protects colominic acid from acid hydrolysis or intramolecular self-cleavage

To investigate whether other factors such as the DMB derivatization temperature and/or the type of acid used for derivatization contribute to the ability to detect higher DP, samples of colominic acid were subjected to DMB derivatization at 50 °C (Fig. 2A), or 4 °C (Fig. 2B). Compared to DMB derivatization at 4 °C, 50 °C derivatization induces the hydrolysis of long polymers. We next tested whether lactonization effectively protects α2-8 sialic acid linkages from acid hydrolysis conditions. Samples that were not subjected to the overnight lactonization step before hydrolysis were primarily detected by HPLC as peaks of Sia oligomers of DP 1–15 (Fig. 2C), with the largest detectable peaks around DP 25. Lactonization produced a noticeable increase in the higher peaks and a decrease in these smaller peaks (Fig. 2D). Thus, lactonization protects higher DP colominic acid during hydrolysis conditions. Furthermore, these results confirm that these oligomers are the result of the degradation of large polySia chains, which after the lactonization step were protected through acid hydrolysis and still detectable up to DP 40.

3.3. Lactonization protects mouse brain polySia structures during release by acid hydrolysis

Our studies with colominic acid illustrate that lactonization protects highly unstable α2-8 sialic acid linkages during acid hydrolysis. Next, we explored whether lactonization protection of α2-8 sialic acid linkages can be used during acid hydrolysis release of polySia chains from biological polysialylated glycoproteins. PolySia is highly expressed by proliferating neural cells during early brain development, and is found throughout neonatal mouse brains. We prepared a whole brain homogenate from postnatal day 1 (P1) mouse pups using pH 8.0 Tris-HCl buffer. We then used organic extraction to remove the abundant lipids from the whole brain homogenate, and treated the protein fraction with proteinase K to produce an aqueous P1 brain glycopeptide sample rich in polysialylated N-glycan structures (Fig. 3A). We used this P1 brain glycopeptide sample to investigate the use of lactonization in the study of mammalian brains glycans.

In samples which were subjected to an initial step of lactonization prior to hydrolysis, we detected eluting polySia structures up to DP ~60 in P1 mouse brains (Fig. 3B). Without an initial lactonization step these large peaks were completely destroyed prior to analysis (Fig. 3C). Additionally, acetic acid hydrolysis of P1 brain glycopeptides generates peaks of mono and oligo sialic acids eluting between 15 and 35 min. These peaks are significantly reduced in area by using lactonization. This suggests that, like colominic acid, the abundance of mono and oligo sialic acids in the non-lactonized sample are the product of hydrolysis of unprotected polySia structures, and that lactonization prior to hydrolysis preserves these structures through release and DMB labeling. While lactonization was previously used to enable MALDI-TOF-Mass Spectrometry of polysialic acid chains [42], here, we use lactonization of polysialylated glycopeptides to improve existing methods in DMB-fluorescence HPLC. This allows for higher resolution fluorescence HPLC characterization of polySia structures in the brain sample. While some degradation likely still occurs, this new approach offers a more accurate glimpse of the endogenous state of brain polysialylation.

3.4. Analysis of dynamic polysialic acid synthesis during early postnatal mouse brain development

Using the lactonization method, we next set out to study a hallmark paradigm of polySia expression and function in the mammalian nervous

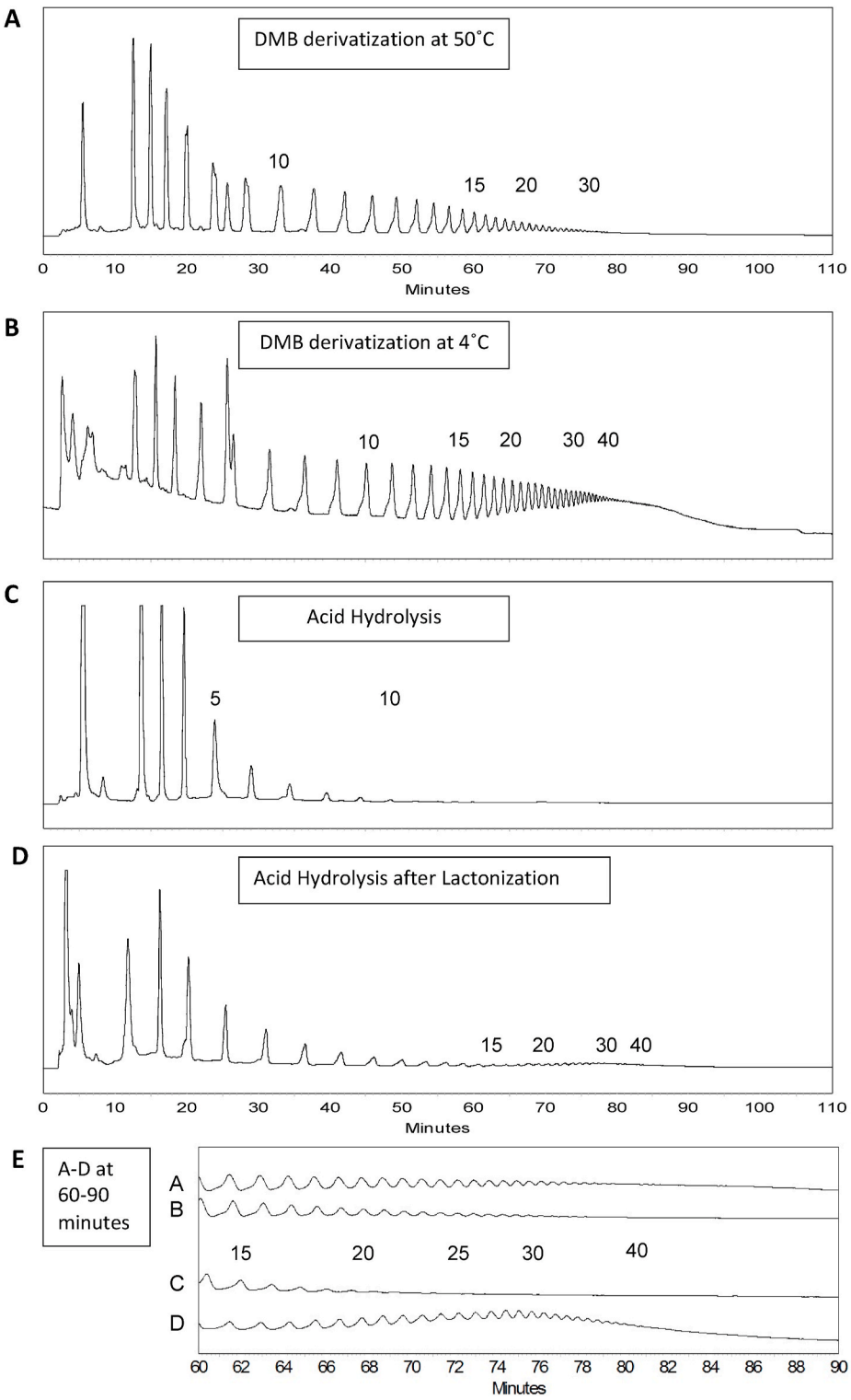

Fig. 2. DMB derivatization of colominic acid also hydrolyzes 2–8 linkages **A)** DMB Derivatization of Colominic acid at 50 °C. **B)** DMB Derivatization of Colominic acid at 4 °C. **C)** Same as B, following mild acid hydrolysis. **D)** Same as C, following lactonization. **E)** Close-up view of all chromatograms from 60 to 90 min.

system. As previously described, polySia expression is highest in the nervous system during embryonic and early postnatal development. As the brain matures, proliferating polysia + neural precursors give rise to the terminally differentiated neural cell types of the adult brain, and eventually polysia + cells are only detected in the specific brain areas of adult neurogenesis, including the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricle.

Brains were collected from mice representing infant, juvenile, and adult stages of neurodevelopment (7 days, 14 days, and 10 weeks of age, respectively). These ages were chosen to represent progressive stages of neural development and to apply our new methods to further the understanding of polySia developmental dynamics. Infant mice showed striking peaks of polySia between DP of 20 and 40, with highest detected peaks even higher (Fig. 4A). Juvenile mice showed similar amounts of

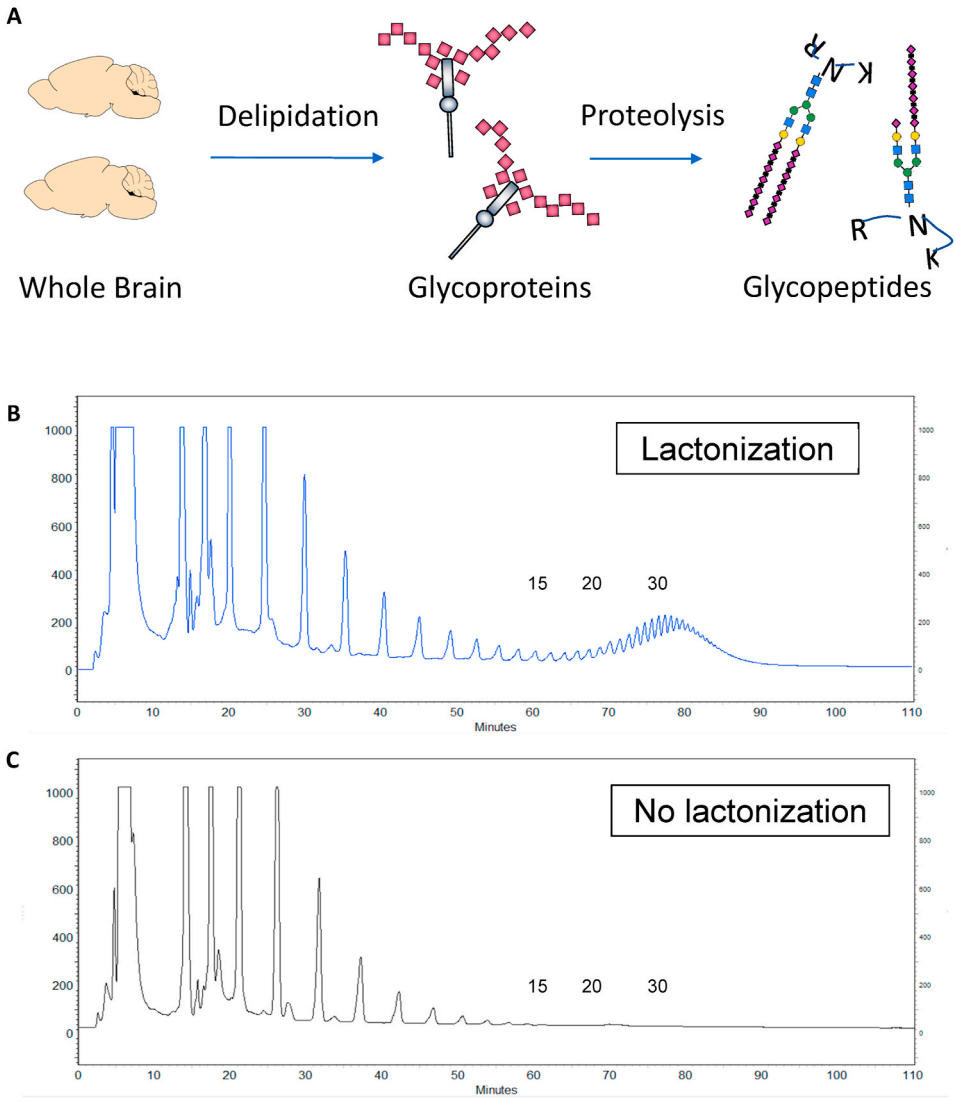

Fig. 3. Lactonization protects high DP polysialic acids during release from mouse brain glycopeptides **A)** Overview of brain glycopeptide preparation: (1) brains are homogenized and delipidated, (2) using proteinase digestion, brain proteins are digested into glycopeptides. **B)** DMB-HPLC analysis of neonate mouse brain glycopeptides after lactonization step. **C)** DMB-HPLC analysis of neonate mouse brain glycopeptides with no lactonization.

polySia up to DP20, but less polymers in the highest size range (Fig. 4B). Adult mice had a smaller, but appreciable amount of the largest polymers (Fig. 4C). Importantly, because lactonization reduces the degradation of large polymers into smaller fragments, this method improves the ability to observe the relative dynamics of polySia chains. Without lactonization, indiscriminate hydrolysis of long polySia chains during sample preparation produces a misleading increase in smaller chains.

These results not only confirm that our method is capable of capturing the dynamic regulation of polySia in mammals, but also offers some insight into the biology of polySia during mouse brain development. Our high-fidelity results emphasize that the abundance of large polymers decreases dramatically with age, disproportionately to the smaller and medium sized polySia chains. The differential developmental profiles of polysialic acid, which we characterized in wild type mice using lactonization-DMB-HPLC, is most likely related to the dynamic developmental regulation of the two polysialyltransferases ST8Sia-II and ST8Sia-IV, which are known to have slightly differing and cooperative activity *in vivo* [25]. The rapid postnatal downregulation of long polySia chains was previously noted by Inoue et al. in chicken embryos, using 4 °C DMB derivatization method [44]. Later work on *St8sia2* and *St8sia4* knockout mice used available DMB-HPLC methods also identified a relatively accelerated decline of longer chains of polysialic acid and implicated the rapid postnatal downregulation of *St8sia2* [19].

Previous studies into polySia function have identified a diverse set of molecular mechanisms through which polysialylated glycans play a role in cellular activity. To name a few, these mechanisms include: physically affecting cell-cell contact, directly interacting with signaling molecules in *cis* or *trans*, and interacting with soluble ligands as either a co-receptor to increase ligand-receptor activity, or a molecular sink to trap ligands and reduce receptor activity [45]. The difference in abundance of larger polySia changes in older mice may be related to medium-shorter chains having a more critical function in adult polySia + cells, such as in hippocampal plasticity, relative to the long-chains roles in developing polySia + cells.

The degree of polymerization (DP) is known to affect the biological functions of polysialic acid. For example, brain derived neurotrophic factor (BDNF), a neural growth factor bind to polySialic acid in a DP-dependent manner [46], suggesting polysialic acid DP can affect the myriad of BDNF-dependent functions, including cell survival, differentiation [47], synaptic plasticity, long-term memory formation [48] as well being associated with neuropsychiatric disorders, such as schizophrenia, major depression and bipolar disorder [49–53]. Additionally, several genetic variations of *ST8SIA2* have also been linked to such

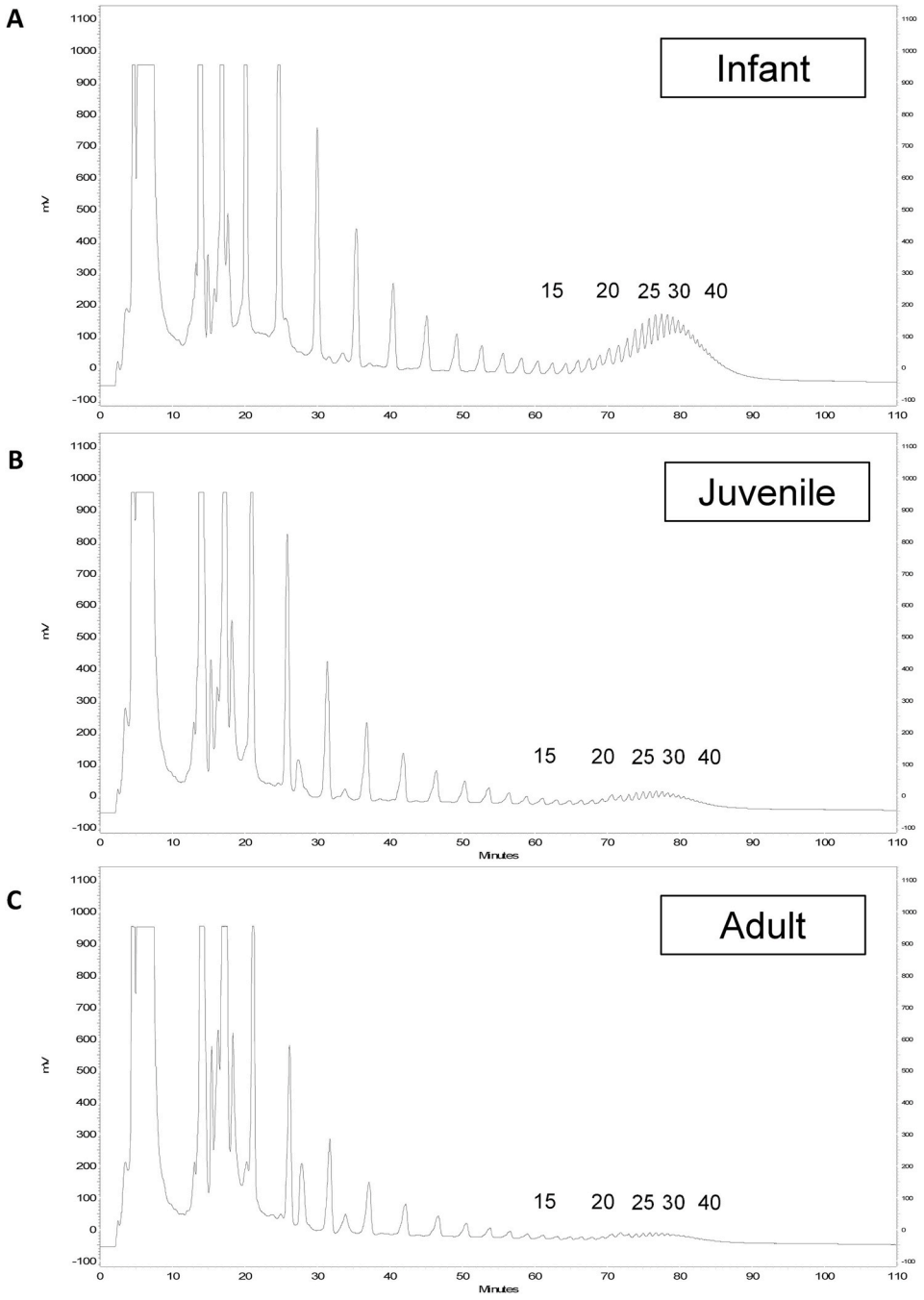

Fig. 4. Lactonization-protection-DMB HPLC long-chain polysia analysis and EndoN-sensitive Neu5Ac analysis during analysis of early postnatal mouse brains. Brains subjected to Lactonization-protection-DMB HPLC analysis: **A)** Infant mouse (P1). **B)** Juvenile mouse (P14). **C)** Adult mouse (10 weeks).

psychiatric disorders, including autism spectrum disorder. In fact, a familial schizophrenia-related ST8Sia-II mutation appears to affect enzyme function by producing shorter polySia chains, highlighting the consequences of dysregulated polySia chain-length in humans. It is possible that, depending on context, cells depend on an abundance of chains of a specific DP for preferential between the many potential functions of polySia. Differential regulation of polySia DP may be involved in the context-specific molecular mechanisms at play for a given polysialylated protein or cell surface. With advances such as the method described this paper, these are all areas that may be approached in the future.

3.5. A complementary enzymatic method to quantitate polySia content

EndoN is a highly specific enzyme from a phage that hydrolyzes 2–8 linkages in chains of **polySia of** DP $\geq$ 7 [54–56] We took advantage of this specificity to quantify total polysialic acid content. We used DMB labeling following EndoN digestion to specifically identify the EndoN sensitive sialic acids. EndoN produces fragments of between 2 and 5 sialic acids [56–59]. [25]. After EndoN treatment was needed to separate the oligomeric products from the glycopeptide sample containing other, non-EndoN-sensitive, sialic acid determinants. To accomplish this, we used a centrifugal filter device with a cutoff of 3000 Da, or ~9 Neu5Ac units, to collect EndoN-sensitive sialic acids before HPLC quantitation.

By digesting a sample with EndoN, labeling the filtered product with

DMB, and comparing the oligomers with a control sample in which EndoN enzyme was omitted, the peaks corresponding with EndoN products can be quantified and summed together to produce a figure representing the amount of polysialic acid in the sample. There is a significant increase in brain mass following birth [19]. To ensure that the dramatic differences in brain mass associated with development does not complicate or affect our result, our glycopeptide extraction and digestion processes were all performed on a scaled weight:weight basis (as described in section 2.3). Additionally, we proceeded through the analytical process by labeling and subsequently injecting an identical quantity of sample relative to original wet brain tissue mass, to minimize any potential consequences of developmental differences on our analytic process.

To illustrate this principle, we once again used colominic acid as a standard. We treated colominic acid with EndoN, collected the products of this reaction via filtration, and subsequently labeled with DMB. HPLC analysis of EndoN-sensitive Neu5Ac released from colominic reveals that all polymers greater than 7 sialic acid residues are enzymatically hydrolyzed into oligomers of DPs 2–5 (Fig. 5A). Omitting EndoN treatment before the filtration step revealed that there were no detectable sialic acid structures less than DP 9 in our colominic acid standard (Fig. 5B). Besides for the intended purpose as a control for enzyme activity, and a background sample for quantitation, this result suggests that in our previous studies, applying various DMB derivatization methods to the same colominic acid sample (Fig. 2A–E), the presence of sialic acid oligomers is purely an experimental artifact, likely secondary to hydrolysis. One of the major improvements of lactonization is a reduction in these artifacts.

Because the oligomers are separated into discrete integrable peaks, we are able to quantitate the amount of colominic acid subject to enzymatic degradation. Using colominic acid as a quantitative standard for EndoN-sensitive Neu5Ac, we determined the polySia content, per brain mass, of the infant, juvenile, and adult brain samples previously used for lactonization length analysis (Table 1). Our results confirm the known trend that polySia content decreases as the brain matures. While other methods do exist to quantitate total amount of polySia, this is a simple and efficient method, and the first quantitative method that takes advantage of the highly specific enzymatic activity of EndoN.

4. Conclusions and perspectives

Here we presented two distinct analytical approaches to characterize both the total amount of polySia in given biological sample containing polysialylated glycan species using EndoN-sensitive Neu5Ac analysis, as well as the relative quantity of polySia chains of a given length within each sample. Although we did not do so, it should also be possible to combine these approaches to specifically calculate the quantitative amount of polySia of a given DP. While our studies are most likely still

Table 1
Polysialic acid content (nmol Neu5Ac/mg tissue) of mouse brain, expressed as nmol of EndoN-sensitive Neu5Ac.

Sample	polySia
Infant	17.85
Juvenile	6.20
Adult	1.17

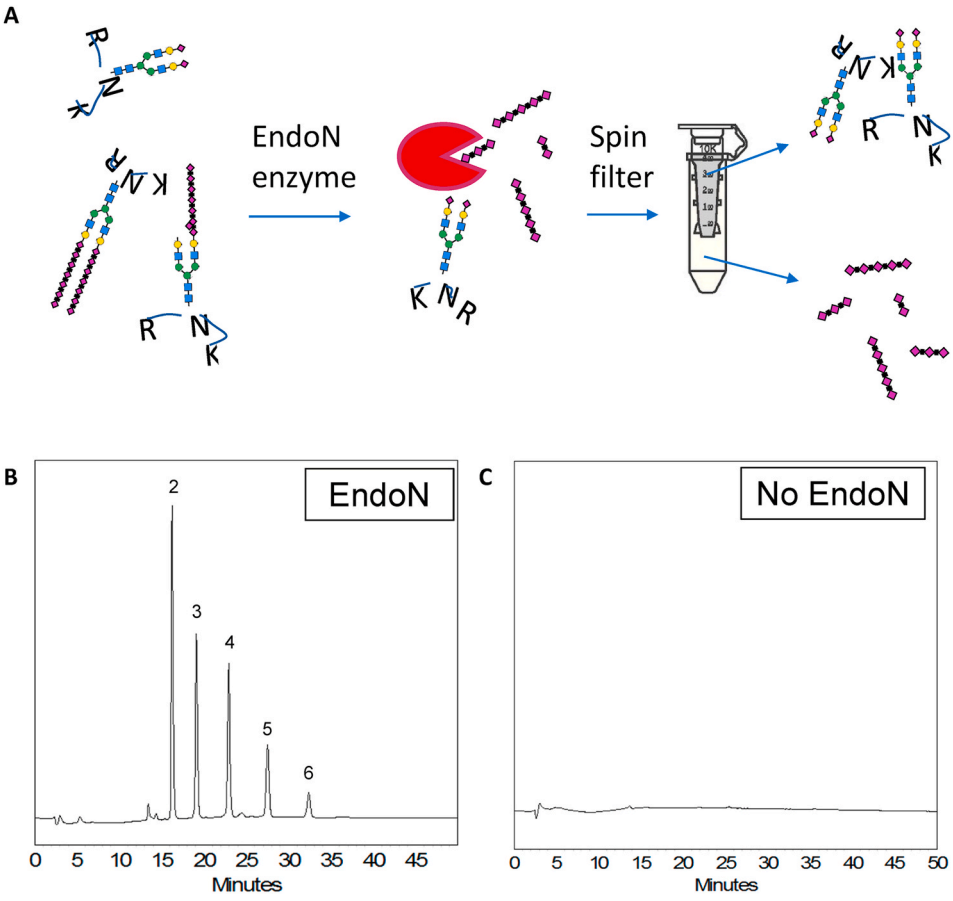

Fig. 5. DMB Analysis of EndoN-sensitive Neu5Ac **A)** Overview of EndoN-sensitive Neu5Ac purification: (1) polysialic acid moieties are digested into oligosialic fragments, which are then (2) separated from glycopeptides and terminal sialic acid structures using a centrifugal filtration device. **B)** HPLC analysis of spin filter flow-through following EndoN digestion of colominic acid. **C)** HPLC analysis of spin filter flow-through of untreated colominic acid.

affected by the intrinsic instability of polySia's α2-8 linkages, we have made progress in more closely revealing the true nature of polySia structures in a biological sample.

In this study, we tested this method of polySa analysis and found that compared to our new lactonization approach, subjecting polysialylated structures to DMB derivatization without lactonization, in both colominic acid and in biological samples, resulted in more hydrolysis. While directly treating samples with DMB reagents may elucidate certain differences between two samples, and qualitatively distinguish them, it is difficult to translate the result into a meaningful representation of polySia structures present before the introduction of random hydrolysis. We applied this new lactonization methods and identified a polySia-developmental profile of mouse brain and found that smaller DP polySia remains abundant as the mouse brain matures, whereas the larger DP polySia decreases. Lastly, we took advantage of the highly specific phage enzyme EndoN, which hydrolyzes 2–8 linkages, and developed a method that allowed us to quantitate amounts of sialic acid in biological samples. While these methods allowed us improved quantitation of polySia, several limitations remain: 1) Lactonization may not prevent all hydrolysis, 2) it is unclear whether DMB-labeling efficiency is the same for DP chains of different length, and 3) the Endo-N method does not account for sialic acids that remain attached to the underlying glycan, as it is not DMB labeled. Studying such an unstable structure will likely remain a technical challenge, but ongoing improvements will continue to refine methodologies to accurately and precisely characterize and quantitate these important molecules, and yield further insight into the regulation of biological processes via polysialylation.

CRediT authorship contribution statement

Michael Vaill: Conceptualization, Methodology, Investigation, Writing – original draft, and Review & Editing, Visualization. **Dillon Y. Chen:** Conceptualization, Methodology, Investigation, Writing – original draft, and Review & Editing, Visualization. **Sandra Diaz:** Conceptualization, Methodology, Investigation. **Ajit Varki:** Conceptualization, Project administration, Supervision, Writing – original draft, and Review & Editing, Resources, Funding acquisition.

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