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AUTHOR(S):

Yaji, Shohei; Manya, Hiroshi; Nakagawa, Naoki; Takematsu, Hiromu; Endo, Tamao; Kannagi, Reiji; Yoshihara, Toru; Asano, Masahide; Oka, Shogo

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Major glycan structure underlying expression of the Lewis X epitope in the developing brain is *O*-mannose-linked glycans on phosphacan/RPTPβ

Shohei Yaji[1], Hiroshi Manya[2], Naoki Nakagawa[1], Hiromu Takematsu[1], Tamao Endo[2], Reiji Kannagi[3], Toru Yoshihara[4, 5], Masahide Asano[4], and Shogo Oka[1]

[1]Department of Biological Chemistry, Human Health Sciences, Graduate School of Medicine, Kyoto University, Kyoto, Japan. [2]Molecular Glycobiology, Research Team for Mechanism of Aging, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo, Japan. [3]Advanced Medical Research Center, Aichi Medical University, Nagakute, Japan. [4]Division of Transgenic Animal Science, Advanced Science Research Center, [5]Research Center for Child Mental Development, Kanazawa University, Kanazawa, Japan.

Running title: Characterization of Lewis X in the developing mouse brain

To whom correspondence should be addressed: Shogo Oka, Ph.D., Kawahara-cho 53, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Tel. & Fax.: +81-75-751-3959, E-mail: shogo@hs.med.kyoto-u.ac.jp

Key words: Lewis X/*O*-mannosyl glycan/phosphacan (RPTPβ)/poly-*N*-acetyllactosamine

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Abstract

Glycosylation is a major protein modification. Although proteins are glycosylated/further modulated by several glycosyltransferases during trafficking form the ER to the Golgi apparatus, a certain glycan epitope has only been detected on a limited number of proteins. Of these glycan epitopes, Lewis X is highly expressed in the early stage of a developing brain and plays important roles in cell-cell interaction. The Lewis X epitope is comprised of a trisaccharide (Galβ1-4 (Fucα1-3) GlcNAc), and a key enzyme for the expression of this epitope is α1,3-fucosyltransferase 9 (Fut9). However, the scaffolding glycan structure responsible for the formation of the Lewis X epitope as well as its major carrier protein has not been fully characterized in the nervous system. We here showed that the Lewis X epitope was mainly expressed on phosphacan/RPTPβ in the developing mouse brain. Expression of the Lewis X epitope was markedly reduced in *β1,4-galactosyltransferase 2* (*β4GalT2)* gene-deficient mice, which indicated that β4GalT2 is a major galactosyltransferase required for the Lewis X epitope. We also showed that the Lewis X epitope almost disappeared due to the knockout of *protein O-mannose β1,2-N-acetylglucosaminyltransferase 1 (POMGnT1)*, an *N*-acetylglucosaminyltransferase essential for the synthesis of *O*-mannosylated glycans, which indicated that the *O*-mannosylated glycan is responsible for presenting the Lewis X epitope. Since *O*-mannosylated glycans on phosphacan/RPTPβ could also present human natural killer-1 (HNK-1), another glycan epitope specifically expressed in the nervous system, our results revealed the importance of *O*-mannosylated glycan chains in the presentation of functional glycan epitopes in the brain.

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Introduction

Glycosylation is one of the most frequent post-translational modifications of proteins (Apweiler et al. 1999). Since cellular glycan structures are diverse, glycan-epitopes can be potentially carried by various proteins. However, a limited number of proteins are modified with specific glycan(s), such as polysialic acids, Lewis X, and HNK-1. Moreover, these "specific glycosylations" have been shown to determine the functionality of the carrier glycoproteins (Kleene and Schachner 2004; Ohtsubo and Marth 2006). Specific glycan modifications on carrier proteins are regulated independently from the expression of the carrier protein per se. However, how such "specific glycosylations" are regulated, remains largely unclear even if the terminal glycosyltransferase forming a specific glycan epitope has been clarified. In other words, the scaffolding glycan structure required for the terminal glycosyltransferase reaction is also important for the expression of a specific glycan epitope. Elucidating the pathways for "specific glycosylation" will provide a deeper insight into the functional and regulatory aspects of protein glycosylation.

The Lewis X epitope, also known as stage-specific embryonic antigen-1 (SSEA-1) or CD15 (Hakomori 1992), plays important roles in cell-cell interactions (Fenderson et al. 1984; Sajdel-Sulkowska 1998). It is known to be both temporally and spatially regulated in the nervous system (Gocht et al 1996); thus, this epitope is a useful maker for neural stem and progenitor cells (Hennen et al. 2011). A recent study suggested that Lewis X is involved in regulating neural stem cells via the Notch signaling pathway (Yagi et al. 2012). The Lewis X epitope is comprised of a trisaccharide (Galβ1-4 (Fucα1-3) GlcNAc), and α1,3-fucosyltransferase 9 (Fut9) is the terminal enzyme for its biosynthesis in the nervous system because almost all Lewis X expression was reported to disappear in Fut9 gene-deficient mice (Kudo et al. 2007). However, crucial information regarding the main glycan structure underlying expression of the Lewis X epitope has yet to be obtained. Moreover, it is still debated whether the Lewis X epitope is presented on phosphacan

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(Allendoerfer et al. 1995) or another chondroitin sulfate proteoglycan (CSPG) (Shimoda et al. 2002). Phosphacan, one of the major soluble CSPGs in the brain, is a secreted-type splicing variant of receptor protein tyrosine phosphatase β (RPTPβ) that is also modified by CS chains (Krueger and Saito 1992; Barnea et al. 1994; Peles et al. 1998). Phosphacan/RPTPβ is expressed by both neuronal and glial cells and involved in numerous neural events including neurite outgrowth, neuronal adhesion and migration, and oligodendrocyte proliferation (Sakurai et al. 1997; Abbott et al. 2008; Lamprianou et al. 2011). As well as the CS chain, phosphacan/RPTPβ carries various glycans such as *N*-glycans, *O*-mannosylated glycans, and keratan sulfate. Previous studies have shown that glycosylation could modulate the function of phosphacan/RPTPβ through the regulation of ligand binding activity, receptor dimerization, and subsequent intracellular signal transduction (Milev et al. 1995; Maeda et al. 2003; Abbott et al. 2008), which reinforce the importance to explore the expression and structure of the Lewis X epitope on phosphacan/RPTPβ.

The *O*-mannose-linked glycan is highly enriched in the brain with 1/3 of all *O*-glycans in the brain being estimated to be *O*-mannose-linked ones (Chai et al. 1999). An *O*-mannosyl tetrasaccharide (Siaα2-3Galβ1-4GlcNAcβ1-2Man) was discovered in bovine peripheral nerve α-dystroglycan (α-DG) (Chiba et al. 1997) and was synthesized by protein *O*-mannosyl transferase 1 (POMT1), POMT2, and protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) (Yoshida et al. 2001; Manya et al. 2004). Mutations in the genes encoding POMT1/2 and POMGnT1 have been shown to cause muscular dystrophies with abnormal α-DG glycosylation, designated as Walker-Warburg syndrome and muscle-eye-brain disease, respectively (Yoshida et al. 2001; Beltran-Valero de Bernabe et al. 2002; van Reeuwijk et al. 2005). Thus, protein *O*-mannosylation has been focused on as a pathological mechanism underlying severe congenital muscular dystrophies associated with neuronal migration defects. However, many *O*-mannosylated proteins including cadherin superfamily molecules have recently been identified (Vester-Christensen et

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al. 2013) and protein *O*-mannosylation is known to be crucial for E-cadherin-mediated cell adhesion (Lommel et al. 2013). In addition, we recently identified the main carrier glycoprotein of the *O*-linked type HNK-1 as phosphacan in developing mouse brains and demonstrated that the HNK-1 epitope was expressed on *O*-mannose-linked glycans (Morise et al. 2014). Therefore, *O*-mannose-linked glycans have more important roles in biological processes than previously thought.

We here attempt to identify the main glycoprotein carrying the Lewis X epitope and the scaffolding glycan structure in developing brains. We found that the Lewis X epitope was mainly expressed on phosphacan/RPTPβ. We also showed that the expression of the Lewis X was markedly reduced in POMGnT1 gene-deficient mice, indicating that a major scaffolding glycan presenting Lewis X is the *O*-mannose-linked glycans.

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Results

Developmental changes in expression of the Lewis X epitope in the mouse brain

The presence of the Lewis X epitope was previously shown to be developmentally regulated in the developing rodent brain (Gocht et al 1996). A previous study also suggested that the epitope occurred on CSPG (Allendoerfer et al. 1995). However, the precise regulation system underlying the characteristic expression of the Lewis X epitope has not yet been determined. To understand its regulatory aspect, we examined developmental changes in Lewis X epitope expression in the mouse brain from embryonic day 16 to postnatal week 10 by utilizing a 73-30 monoclonal antibody (mAb) specific to the Lewis X epitope (Mitsuoka et al. 1998; Ohmori et al. 2001). We only detected the Lewis X signal at very high molecular weights in both the soluble and membrane fractions (Figure 1A). Consistent with the findings of a previous study, Lewis X signal on immunoblots was enhanced and sharpened by the chondroitinase ABC (CHase ABC) treatment, indicating that the Lewis X epitope occurs on CSPG (Allendoerfer et al. 1995); however the identity of the main carrier protein has yet to be confirmed (Allendoerfer et al. 1995; Shimoda et al. 2002). The Lewis X epitope level peaked at postnatal week 2 (P2w) to P4w and a strong signal was detected from P1w to P4w in both the soluble and membrane fractions. These results suggested that Lewis X epitope expression is tightly regulated during brain development. Similar immunoblotting analyses of the P2w brain were performed using other Lewis X-recognizing antibodies, anti-SSEA-1 and anti-CD15. Both of these two antibodies gave a single prominent band over 250 kDa, which was similar to the immunoblot with 73-30 mAb (Figure 1B). Moreover, we could observe some additional Lewis X-positive bands on the blot under long exposure conditions (Figure 1B, right panel). These results indicated that there are one major and several minor carrier proteins, which underlie the expression of the Lewis X epitope in the developing brain. The migration position of the Lewis X band detected in the soluble fraction was similar to that in the membrane fraction, suggesting that Lewis X-carrying proteins in both fractions may have

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similar molecular natures. Regarding the characterization of the Lewis X epitope, the soluble fraction has several advantages compared to the membrane fraction when we perform biochemical experiments, such as the strong Lewis X immunoreactivity and high solubility to aqueous solutions. Therefore, we initially used the P2w-soluble fraction in subsequent experiments, and the obtained information was utilized in the later analysis of the Lewis X carrier in the membrane fraction.

Characterization of the Lewis X epitope

Lewis X could be further modified by a sialic acid on its galactose residue and thus exist as sialyl Lewis X (Siaα2-3Galβ1-4 (Fucα1-3) GlcNAc), which was no longer recognized by the 73-30 mAb (Mitsuoka et al. 1998). To assess the amount of Lewis X modified by sialic acids in the brain, we carried out an immunoblotting analysis of a sialidase-treated P2w brain soluble fraction. The sialidase treatment slightly augmented the signal intensity of the band over 250 kDa, which suggested that the CSPG also carries sialyl Lewis X (Supplementary data, Figure S1). A recent mass spectrometric analysis revealed that, in the mouse brain, the existence of sialyl Lewis X was quite small among the entire Lewis X-related structures (Parry et al. 2007). Likely due to the low expression level of sialyl Lewis X, we did not observe any additional Lewis X-reactive bands after the sialidase treatment (Supplementary data, Figure S1).

The Lewis X epitope can occur on various glycans. Lewis X was previously reported to be presented on the *N*-glycan of tenascin-C (Hennen et al. 2011) and low density lipoprotein receptor-related protein-1 (LRP-1) (Hennen et al. 2013). It could also be presented on the *O*-glycan of P-selectin glycoprotein ligand-1 (PSGL-1) (Moore et al. 1996). The HNK-1 epitope is also expressed on *N*-glycans and *O*-glycans in the nervous system. Therefore, we characterized the Lewis X epitope by comparing it with the HNK-1 epitope. When the P2w-soluble fraction was digested with the peptide *N*-glycosidase F, HNK-1 positive bands at

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approximately 160 and 180 kDa were eliminated (Figure 1C). In contrast, digestion with the peptide *N*-glycosidase F did not affect either the HNK-1 or Lewis X signal over 250 kDa (Figure 1C). This result indicated that P2w Lewis X was not expressed on *N*-glycans, which is a similar property to the HNK-1 epitope over 250 kDa. We recently reported that the HNK-1 carrier protein present over 250 kDa was the CSPG, phosphacan, and the HNK-1 epitope was expressed on *O*-mannosylated glycans (Morise et al. 2014), which suggested that the Lewis X epitope may be expressed on the *O*-mannosylated glycans of phosphacan/RPTPβ.

Involvement of poly-N-acetyllactosamine in Lewis X expression

A previous study reported that the Lewis X epitope could be presented on the poly-*N*-acetyllactosamine (polyLacNAc) units of glycans in the brain (Allendoerfer et al. 1995). Then, we compared the Lewis X positive band with the polyLacNAc signal. Tomato lectin, *Lycopersicon esculentum* lectin (LEL) is specific to polyLacNAc (Merkle and Cummings 1987), and, thus, was used for this comparison. Lectin-blotting by LEL resulted in the detection of a high molecular weight band similar to the Lewis X band (Figure 1D). Therefore, we investigated whether these epitopes occurred on the same glycoprotein(s). The high molecular weight Lewis X signal was lectin-precipitated with LEL (Figure 1D), suggesting that Lewis X could be presented on the polyLacNAc chain of CSPG with a high molecular weight.

Phosphacan is the carrier protein for the epitope

As described above, we previously identified the carrier protein of *O*-mannosylated HNK-1 as phosphacan/RPTPβ (Morise et al. 2014), and the carrier protein of Lewis X also showed a similar molecular weight (Figure 1C). To examine whether the carrier protein was phosphacan, we performed immunoblot analyses of the P2w-soluble fraction after CHase ABC treatment using anti-phosphacan and anti-aggrecan antibodies because aggrecan was found to have the

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most similar molecular weight to phosphacan among the CSPGs detected in the brain (Figure 2A). Tenascin-C was also analyzed because it could bear the Lewis X epitope and had the high molecular weight (over 250 kDa) (Hennen et al. 2011). The molecular weight of tenascin-C was distinguishably smaller than that of phosphacan and aggrecan (Figure 2A). Moreover, in contrast to phosphacan and aggrecan, tenascin-C did not show the lower shift in the molecular weight by CHase ABC treatment (Figure 2A). Based on these results, tenascin-C was excluded from the candidates of the major carrier of the Lewis X epitope. Since the comparative analysis focusing on the molecular weight could not distinguish between phosphacan and aggrecan, developmental alterations in the expression of these two CSPGs were assessed. The phosphacan signal peaked from P1w to P4w, whereas the aggrecan signal peaked from P4w to P10w (Figure 2B). These results indicated that phosphacan may coincide with the Lewis X profile.

Then, phosphacan and aggrecan were immunoprecipitated to examine the simultaneous immunoprecipitation of the Lewis X signal. The phosphacan-immunoprecipitated (bound) fractions contained Lewis X signals in both the P2w-soluble and membrane fractions (Figure 3A), whereas Lewis X signals were not detected in the aggrecan-immunoprecipitated fractions (Figure 3C). These results showed that phosphacan was the carrier protein of the Lewis X epitope, which was consistent with the findings of a previous study in which the immunoprecipitation by the anti-Lewis X mAb FORSE1 resulted in the detection of phosphacan in the precipitate (Allendoerfer et al. 1995). Moreover, we examined the Lewis X carrier in the late developmental stage (P10w), where the expression of phosphacan decreased while that of aggrecan conversely increased (Figure 2B). The immunoprecipitaion experiments using P10w samples yielded the same results as those using P2w samples (Figure 3, B and D). In addition, the phosphacan immunoreactivity was detected in the LEL-precipitated fraction (Figure 3E). These findings firmly supported the notion that phosphacan was the major carrier of the Lewis X and polyLacNAc chain throughout developmental periods examined in this

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study. The immunoreactivity of Lewis X was observed at a slightly higher molecular weight than that of phosphacan (Figure 3, A and B) but the migration position of the Lewis X immunoreactivity corresponded to that of LEL-precipitated phosphacan (Figure 3E). Furthermore, another immunoprecipitation analysis using the anti-Lewis X antibody revealed that the Lewis X-carrying phosphacan in the precipitated fraction showed almost the same migration position as the Lewis X-reactive band, which was slightly higher than the apparent molecular weight of the total phosphacan in the input fraction (Figure 3F). Thus, these results suggested that phosphacan may be a diversely glycosylated protein and also that the proportion of Lewis X- and polyLacNAc-carrying phosphacans may be relatively small among phosphacan molecules.

Identification of the Lewis X biosynthetic pathway

Since the Lewis X epitope is comprised of a trisaccharide (Galβ1-4 (Fucα1-3) GlcNAc) and might be presented on polyLacNAc chain(s), we attempted to identify the galactosyltransferase responsible for the biosynthesis of Lewis X. Galactosylation of the *N*-acetyllactosamine and polyLacNAc can be achieved by β4GalT family enzymes. Of those, β4GalT1-4 could be involved in the biosynthesis of polyLacNAc (Ujita et al. 1998; Ujita et al. 1999; Chang et al. 2013). In addition, a previous study using *in vitro* assay systems demonstrated that β4GalT1 and 2 showed higher activities toward a polyLacNAc acceptor substrate than other β4GalTs, and the expression of β4GalT2 was higher than that of β4GalT1 in the brain (Sasaki et al. 2005). Therefore, we hypothesized that β4GalT2 could be responsible for the biosynthesis of polyLacNAc and the Lewis X epitope in the developing brain. When the *β4GalT2* knockout (KO) P2w-soluble fraction was analyzed for polyLacNAc expression using a LEL lectin blot, a marked reduction in the epitope was detected (Figure 4A). This is the first study that demonstrates the involvement of β4GalT2 in the biosynthesis of polyLacNAc *in vivo*. Lewis X immunoreactivity was also reduced in the *β4GalT2* KO mice,

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indicating that β4GalT2 has an important role in the expression of the Lewis X epitope and suggesting the possibility that the Lewis X epitope is mainly present on the polyLacNAc chain(s) in the developing brain. It should be noted that Lewis X and LEL signals were marginally detected in the *β4GalT2* KO, suggesting the presence of other β4GalT(s) responsible for polyLacNAc in the brain.

Our results indicated that the Lewis X epitope was not on *N*-glycans (Figure 1C). As well as from *N*-glycans and GalNAc-initiated *O*-glycans, polyLacNAc could be extended from *O*-mannose-linked glycans because the keratan sulfate chain, a sulfated-derivative of polyLacNAc, has shown to be elongated from *O*-linked mannose residues (Krusius et al. 1986). Moreover, the HNK-1 epitope was previously detected on *O*-mannosylated glycans of phosphacan/RPTPβ in a neuroblastoma cell line (Abbott et al. 2008) and the developing mouse brain (Morise et al. 2014). Therefore, we determined whether the Lewis X epitope was presented on *O*-mannosyl glycan(s). *O*-mannosyl glycans are known to be biosynthesized by the first enzyme POMT (Beltran-Valero de Bernabe et al. 2002), and *N*-acetylglucosamine (GlcNAc) could be transferred to *O*-mannose by POMGnT1 (Yoshida et al. 2001). We prepared the P3.5w soluble and membrane fractions from the *POMGnT1* KO brain. Lewis X immunoreactivity was markedly reduced in both the soluble and membrane fractions of the *POMGnT1* KO brain (Figure 4B). Moreover, the LEL-reactive polyLacNAc signal was concomitantly reduced in *POMGnT1* KO (Figure 4B). The loss of these signals also coincided with a shift in the phosphacan band to a lower molecular weight on immunoblots, indicating that almost all phosphacan molecules had substantial amounts of *O*-mannosylated glycans, which potentially contained *N*-acetyllactosamine structures responsible for the Lewis X epitope. These results suggested that the expression of Lewis X on phosphacan may require the specific scaffold(s), such as polyLacNAc, which is also expressed on a subpopulation of phosphacans.

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Discussion

In the present study, we revealed that the major Lewis X epitope in the developing brain was on an *O*-mannosylated glycan synthesized by β4GalT2 and POMGnT1, which was carried by phosphacan/RPTPβ. We further obtained the results suggesting that Lewis X was present on the polyLacNAc chain. The Lewis X epitope in the nervous system was shown to be expressed on *N*-glycans of several glycoproteins such as tenascin-C (over 250 kDa), L1 cell adhesion molecule (L1-CAM, approximately 140 kDa), and LRP-1 (515 kDa α-subunit and 85 kDa β-subunit) (Hennen et al. 2011; Hennen et al. 2013), and also on *O*-mannosylated glycans of CD24 (approximately 30 kDa) or α-DG (100-120 kDa) (Smalheiser et al. 1998; Bleckmann et al. 2009). However, these did not appear to be the main carrier proteins over phosphacan/RPTPβ because the Lewis X epitope on the main carrier protein in the developing mouse brain did not disappear following the peptide *N*-glycosidase F treatment and was also detected over 250 kDa (Figure 1).

The characteristic expression of the Lewis X epitope on phosphacan, especially on its specific glycoform in early developmental stages, suggests that Lewis X is one of key determinants of the functionality of phosphacan. Phosphacan/RPTPβ has bidirectional effects on neurite outgrowth, which induce promotion and inhibition of neurite extension in a context-dependent manner (Sakurai et al. 1997; Inatani et al. 2001). Although the neurite outgrowth-regulating activity of phosphacan has been shown to in part depend on glycan modifications such as CS chains (Inatani et al. 2001), the role of Lewis X is not well examined. However, considering the recent study showing that glial CD24 regulates neurite outgrowth through a Lewis X-dependent interaction with the neuronal receptors that evoke neuritogenesis (Lieberoth et al. 2009), it is possible that the Lewis X structure may modulate the phosphacan-induced neurite outgrowth by affecting the adhesive property of phosphacan. Therefore, Lewis X, as well as CS chains, is likely to act as a modulator of the complex function of phosphacan in neuritogenesis. In addition, polyLacNAc is a unique structure

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extended from *N*- and *O*-glycans, but our result from *POMGnT1* KO mice clearly showed that polyLacNAc was predominantly present in the *O*-mannose-linked form in the mouse brain (Figure 4B). Since polyLacNAc is often found on β1,6-branched GlcNAc (Togayachi et al. 2010), the newly identified *O*-mannosylated polyLacNAc could be linked to β1,6-branched GlcNAc on mannose. This β1,6-branched GlcNAc linkage was biosynthesized by *N*-acetylglucosaminyltransferase-IX (GnT-IX) after a POMGnT1 reaction in the brain. GnT-IX was shown to be responsible for enhancing the expression of *O*-mannosylated glycans on RPTPβ and promoting the dimerization of the molecule, which ultimately attenuated phosphatase activity (Abbott et al. 2008). Thus, *O*-mannosylated polyLacNAc and the Lewis X epitope modified on it appeared to be involved in regulating phosphatase activity of RPTPβ. Functional evaluation of such glycosylation-specific functions of phosphacan/RPTPβ is an important topic for the future study, where the biosynthetic pathways of Lewis X and polyLacNAc in the brain provided here could be useful information.

Previous studies showed that Lewis X epitope levels were developmentally regulated during brain development (Gocht et al. 1996; Hennen et al. 2011). We obtained a similar result that showed the dynamic developmental regulation of this epitope (Figure 1A). The fucosyltransferase responsible for the Lewis X biosynthesis was shown to be Fut9 in the nervous system (Nishihara et al. 2003; Kudo et al. 2007). Since the loss of *Fut9* in mice resulted in increased anxiety-like behavior(s) (Kudo et al. 2007), regulating the Lewis X epitope in the developing brain may be crucial for emotional behavior. In the present study, we demonstrated that Lewis X epitope expression was also regulated by *β4GalT2*, *POMGnT1,* and *phosphacan/RPTPβ*. The phenotype(s) commonly observed in these KO mice may indicate the function of Lewis X on phosphacan/RPTPβ. It remains unknown whether these KO mice develop similar behavioral phenotypes. From this point of view, a clear understanding of the whole glycosylation pathway of a specific glycan epitope is important for elucidating its function because the epitope could be presented on multiple linkages to proteins and regulated

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by multiple enzymes.

The most studied *O*-mannosylated glycoprotein in mammals is α-DG. α-DG associates with its extracellular ligands, such as laminin, agrin, and pikachurin via its *O*-mannosylated glycan (Chiba et al. 1997; Sato et al. 2008), and defects in the *O*-mannosylation of α-DG were shown to cause severe muscular dystrophies, some of which exhibited brain abnormalities. Accumulating evidence has indicated that the unique glycan chains containing disaccharide repeats composed of xylose and glucuronic acid, which is extended via the phosphodiester linkage from the phosphorylated *O*-linked mannose on α-DG, play a pivotal role in the α-DG-ligand interaction (Yoshida-Moriguchi et al. 2010; Inamori et al. 2012). This type of *O*-mannose-linked glycans, so-called post-phosphoryl modification, appeared to be specific for α-DG. Reductions in the molecular weight of phosphacan were observed in the *POMGnT1* KO mouse brain (Figure 4B), which indicated that phosphacan possesses many *O*-mannosylated glycans. However, the post-phosphoryl modification seems to be absent in phosphacan because a recent study showed that the glycosylation state of phosphacan was unchanged in the myodystrophy (myd) mouse, in which like-acetylglucosaminyltransferase (LARGE), a responsible enzyme for the extension of the disaccharide repeats in the post-phosphoryl modification, was mutated (Dwyer et al. 2012). The *O*-mannosylated glycans of phosphacan are instead used as the base structure for the expression of Lewis X and HNK-1 epitopes (Morise et al. 2014). Therefore, *O*-mannosylated glycans are important for the presentation of functional glycan epitopes as well as post-phosphoryl modifications in the nervous system. Combined with the findings of a recent study in which protein *O*-mannosylation was found to be crucial for E-cadherin-mediated cell adhesion (Lommel et al. 2013), *O*-mannose-linked glycans have more important roles in biological processes as well as in pathological processes.

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Materials and methods

Materials

The monoclonal antibody (mAb) specific to the Lewis X epitope (clone 73-30) was established from BALB/c mice immunized with *Salmonella Minnesota* coated with a purified glycolipid having the terminal Lewis X epitope (the structure of the antigenic glycolipid was $III^3Fuc\alpha nLc_4Cer$), and its specificity against Lewis X and related glycans was reported previously (Mitsuoka et al. 1998; Ohmori et al. 2001). Anti-SSEA-1 mAb (clone MC480, BD pharmingen) and anti-CD15 mAb (clone 80H5, Beckman Coulter) were also used to detect the Lewis X epitope. Anti-phosphacan/RPTPβ polyclonal antibodies (pAb) were raised in rabbits against recombinant full-length phosphacan, which was expressed and purified from COS-1 cells. COS-1 cells were purchased from American Type Culture Collection (ATCC CRL-1650) (Gluzman 1981). The HNK-1 hybridoma cell line was purchased from the American Type Culture Collection. The following reagents were obtained from commercial sources; antibodies against aggrecan (AB1031) (Millipore), Tenascin-C (clone MTn-12) (Sigma), actin (clone EP184E) (Millipore), N-cadherin (clone 32) (BD Transduction Laboratories), biotinylated *Lycopersicon esculentum* (Tomato) lectin (Vector Laboratories), the HRP-conjugated polyclonal antibodies, anti-mouse IgG, anti-rabbit IgG (Invitrogen), and anti-mouse IgM (Thermo), Protein G-conjugated Sepharose (GE Healthcare), and streptavidin-conjugated agarose (Thermo).

Mice

Knockout (KO) mice for *beta-1, 4-galactosyltransferase2* (*β4GalT2)* and *POMGnT1* were described previously (Miyagoe-Suzuki et al. 2009; Yoshihara et al. 2009). All the animal experiments were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan

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and approved by the Committees on Animal Experimentation of Kanazawa University, Kyoto University, and Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology.

Preparation of soluble and membrane fractions from the mouse brain

The whole brains of embryonic day 16, postnatal weeks 1, 2, 4, and 10 wild-type mice, postnatal week 2 *β4GalT2* KO mice, and postnatal week 3.5 *POMGnT1* KO mice were homogenized with a polytron homogenizer in 9 volumes of 20 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail (Nacalai Tesque). The homogenates were centrifuged at 1,000 × g for 10 min at 4˚C to remove nuclei and then centrifuged at 105,000 × g for 1 h at 4˚C. The supernatants were then used as soluble fractions. The resulting pellets were resuspended in Tris-buffered saline (TBS) containing 1% Triton X-100, and protease inhibitors, and membrane proteins were then extracted with sonication. Extracts were centrifuged at 105,000 × g for 1 h at 4˚C and the supernatants were used as membrane fractions.

Glycosidase digestion

In the chondroitinase ABC (CHase ABC) treatment, the proteins of mouse brain-soluble and membrane fractions were precipitated by ethanol using the following protocol: 2.5 volumes of 100% ethanol were added to the soluble fractions and incubated for 15 min at -20˚C. Proteins were recovered by centrifugation at 13,000 × g for 10 min at 4˚C. The pellets were washed with 70% ethanol and centrifuged again at 13,000 × g for 5 min at 4˚C. These pellets were suspended with 100 μl of 100 mM Tris-HCl buffer (pH 7.4) containing 30 mM sodium acetate, 50 mM EDTA, and protease inhibitors. CHase ABC (Seikagaku Corporation) was added (300 mU/ml at the final concentration) to the suspension and incubated for 3 h at 37˚C. For the *N*-glycosidase F digestion, proteins treated with the CHase ABC were precipitated by ethanol. The pellets were dissolved and denatured with 50 μl of

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phosphate-buffered saline (PBS) containing 0.5% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, and 20 mM EDTA for 5 min at 100˚C. Samples were then diluted with 4 volumes of PBS. Nonidet P-40 (0.5% at the final concentration) and peptide *N*-glycosidase F (Roche, 20 U/ml at the final concentration) were added and samples were incubated for 16 h at 37˚C. For the sialidase digestion, the CHase ABC treated samples were precipitated by ethanol. The pellets were dissolved in 50 mM phosphate buffer (pH 6.8). Sialidase from *Arthrobacter ureafaciens* (Nacalai Tesque, 50 U/ml at the final concentration) was added to the samples and incubated for 16 h at 37℃. In the initial step, 200 μg of protein was used in all the experiments except for immunoprecipitation experiments, where 500 μg of protein was used.

Immunoprecipitation and lectin-precipitation

The primary antibody (10-20 μg/ml at the final concentration) or biotinylated *Lycopersicon esculentum* lectin (10 μg/ml at the final concentration) was added to the 200 μl of glycosidase-treated soluble and membrane fractions, which contain 500 μg of protein. After addition of 25 μl of Protein G-conjugated Sepharose for immunoprecipitation or streptavidin-conjugated agarose for lectin-precipitation, the samples were incubated with gentle shaking at 4˚C for 16 h. The beads were subsequently recovered by centrifugation (400 × g for 2 min) and then washed three times with an excess volume of TBS containing 0.1% Tween 20. The beads were boiled in Laemmli sample buffer and eluted proteins were subjected to SDS-PAGE.

SDS-PAGE, immunoblotting, and lectin-blotting analyses

Proteins (30 μg of protein in each lane) in soluble and membrane fractions were separated on a 7% gel with SDS-PAGE and then transferred onto nitrocellulose membranes. In the case of immunoblotting, membranes were incubated with primary antibodies after blocking with 5% skimmed milk in PBS containing 0.05% Tween 20, followed by HRP-conjugated

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secondary antibodies. In the case of lectin-blotting, membranes were incubated with biotinylated lectin after blocking with 3% BSA in PBS containing 0.05% Tween 20, followed by the incubation with the VECTASTAIN ABC Kit (Vector Laboratories). Conjugated HRP was detected with the chemiluminescent substrate Super Signal West Pico (Thermo) and the LAS3000 image analyzer (Fujifilm).

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Abbreviations

The abbreviations used are: α-DG, α-dystroglycan; Fut9, α1,3-fucosyltransferase 9; β4GalT, β1,4-galactosyltransferase; CHase ABC, chondroitinase ABC; GnT-IX, *N*-acetylglucosaminyltransferase-IX; HNK-1, human natural killer-1; LEL, *Lycopersicon esculentum* lectin; mAb, monoclonal antibody; pAb, polyclonal antibody; polyLacNAc, poly-*N*-acetyllactosamine; POMGnT1, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1; POMT, protein *O*-mannosyl transferase; RPTPβ, receptor protein tyrosine phosphatase β.

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Legends to figures

Figure 1. Developmentally regulated expression of the Lewis X epitope in the mouse brain. (**A**) The whole brain from mice at the indicated ages (E, embryonic days; P, postnatal weeks) were homogenized, and soluble and membrane fractions were prepared as shown in the **Materials and methods**. Each fraction was digested with chondroitinase ABC (CHase ABC) and samples were subjected to immunoblotting with the anti-Lewis X mAb. (**B**) CHase ABC-digested soluble and membrane fractions prepared from the P2w brain were immunoblotted with the anti-Lewis X, anti-SSEA-1, and anti-CD15 mAbs (left). The immunoblots with anti-CD15 under long exposure conditions were also shown (right). (**C**) The soluble fraction from the P2w whole brain was successively digested with CHase ABC and *N*-glycosidase F, and samples were subjected to immunoblotting with the anti-HNK-1 mAb and anti-Lewis X mAb. Anti-actin was used as a loading control. (**D**) The P2w brain-soluble fraction was digested with CHase ABC and lectin-blotted or immunoblotted with LEL or the anti-Lewis X mAb, respectively (left). The CHase ABC-treated P2w brain-soluble fraction was lectin-precipitated with LEL. Proteins in the input fraction (I) and lectin-precipitated fraction (P) were immunoblotted or lectin-blotted with the anti-Lewis X mAb or LEL, respectively (right).

Figure 2. Phosphacan shows highly consistent biochemical properties with the Lewis X epitope. (**A**) The P2w brain-soluble fraction was digested with CHase ABC and immunoblotted with anti-phosphacan/RPTPβ pAb, anti-aggrecan pAb, and anti-tenascin-C mAb. (**B**) Soluble and membrane fractions from developing mice (age E16 to P10w) were digested with CHase ABC, and samples were subjected to immunoblotting with anti-phosphacan/RPTPβ pAb (top) and anti-aggrecan pAb (bottom).

Figure 3. Characterization of the major carrier protein of Lewis X in the developing

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mouse brain. (**A** and **B**) CHase ABC-digested soluble and membrane fractions prepared from P2w (**A**) or P10w (**B**) brains were subjected to immunoprecipitation against anti-phosphacan/RPTPβ pAb. Input (I) and precipitated (P) fractions were subjected to immunoblotting with the anti-Lewis X mAb and anti-phosphacan/RPTPβ pAb. (**C** and **D**) CHase ABC-digested soluble fractions prepared from P2w (**C**) or P10w (**D**) brains were subjected to immunoprecipitation against anti-aggrecan pAb. Input (I) and precipitated (P) fractions were subjected to immunoblotting with the anti-Lewis X mAb and anti-aggrecan pAb. (**E** and **F**) The CHase ABC-treated P2w brain-soluble fraction was lectin-precipitated with LEL (**E**) or immunoprecipitated using the anti-Lewis X mAb (**F**). Input (I) and precipitated (P) fractions were immunoblotted with the anti-Lewis X mAb and anti-phosphacan/RPTPβ pAb (**E** and **F**) or lectin-blotted with LEL (**E**).

Figure 4. The Lewis X expression is markedly reduced in both *β4GalT2* and *POMGnT1* gene-deficient mice. (**A**) The P2w whole brain soluble fraction of wild-type (WT) and *β4GalT-2*$^{-/-}$ mice was digested with CHase ABC, and samples were subjected to immunoblotting with the anti-Lewis X mAb and lectin-blotting with LEL. Anti-actin was used as a loading control. (**B**) The soluble and membrane fractions from the P3.5W whole brains (wild-type (+/+), *POMGnT1* heterozygous (+/-), and *POMGnT1* KO (-/-)) were digested with CHase ABC and samples were subjected to immunoblotting with the anti-Lewis X mAb and anti-phosphacan/RPTPβ pAb or lectin-blotting with LEL. Anti-actin pAb and anti-N-cadherin mAb were used as loading controls.

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Figure 1

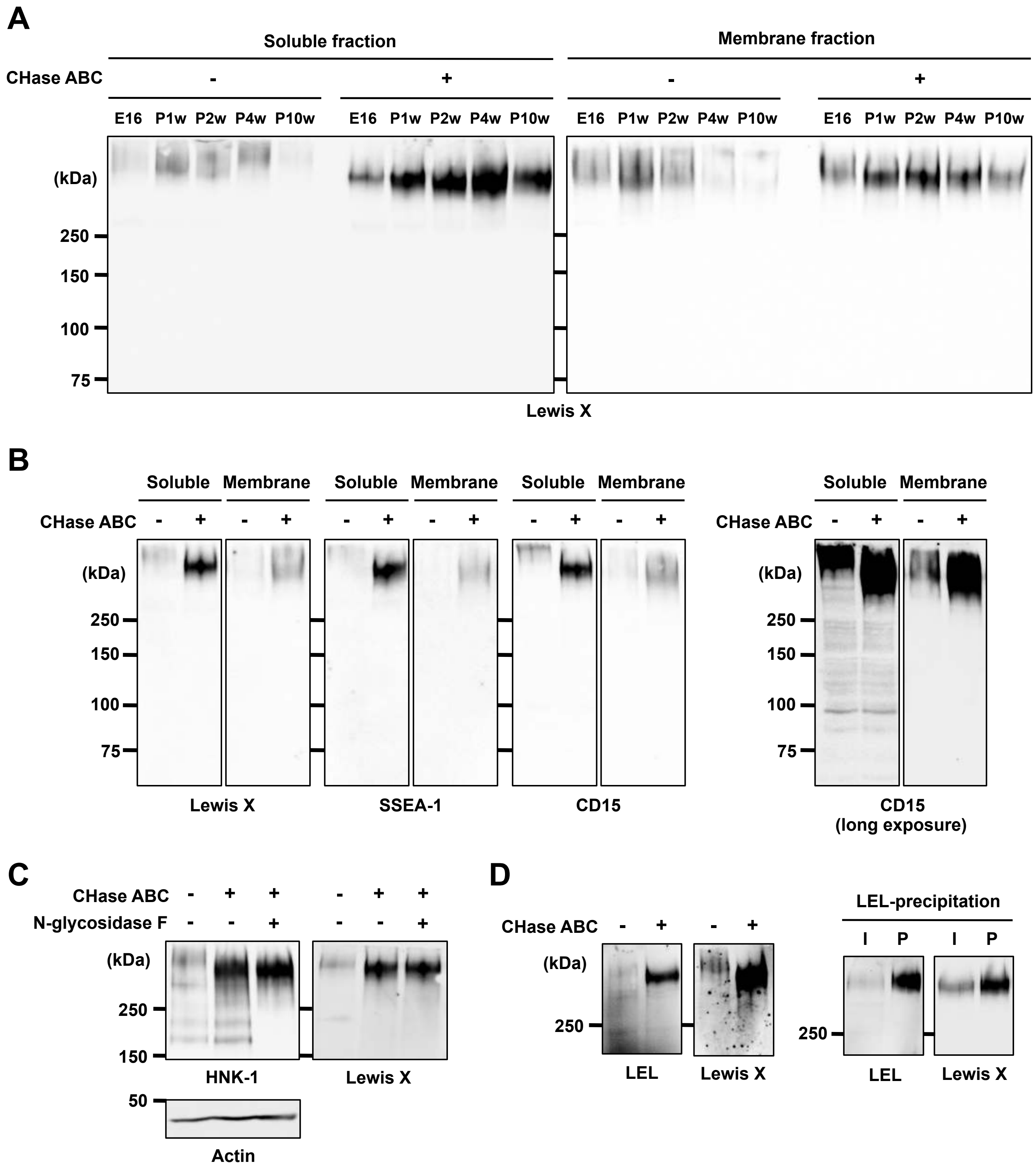

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Figure 2

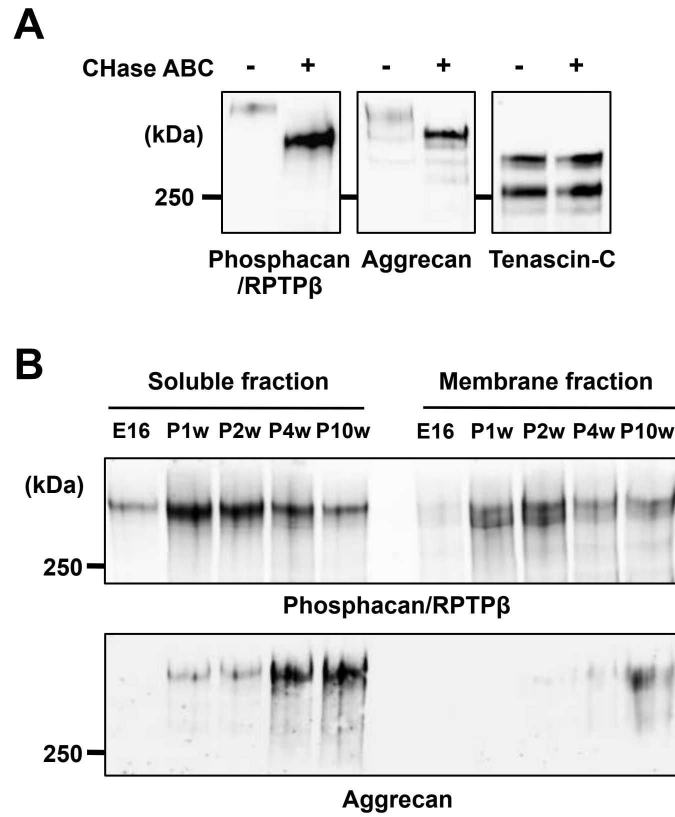

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Figure 3

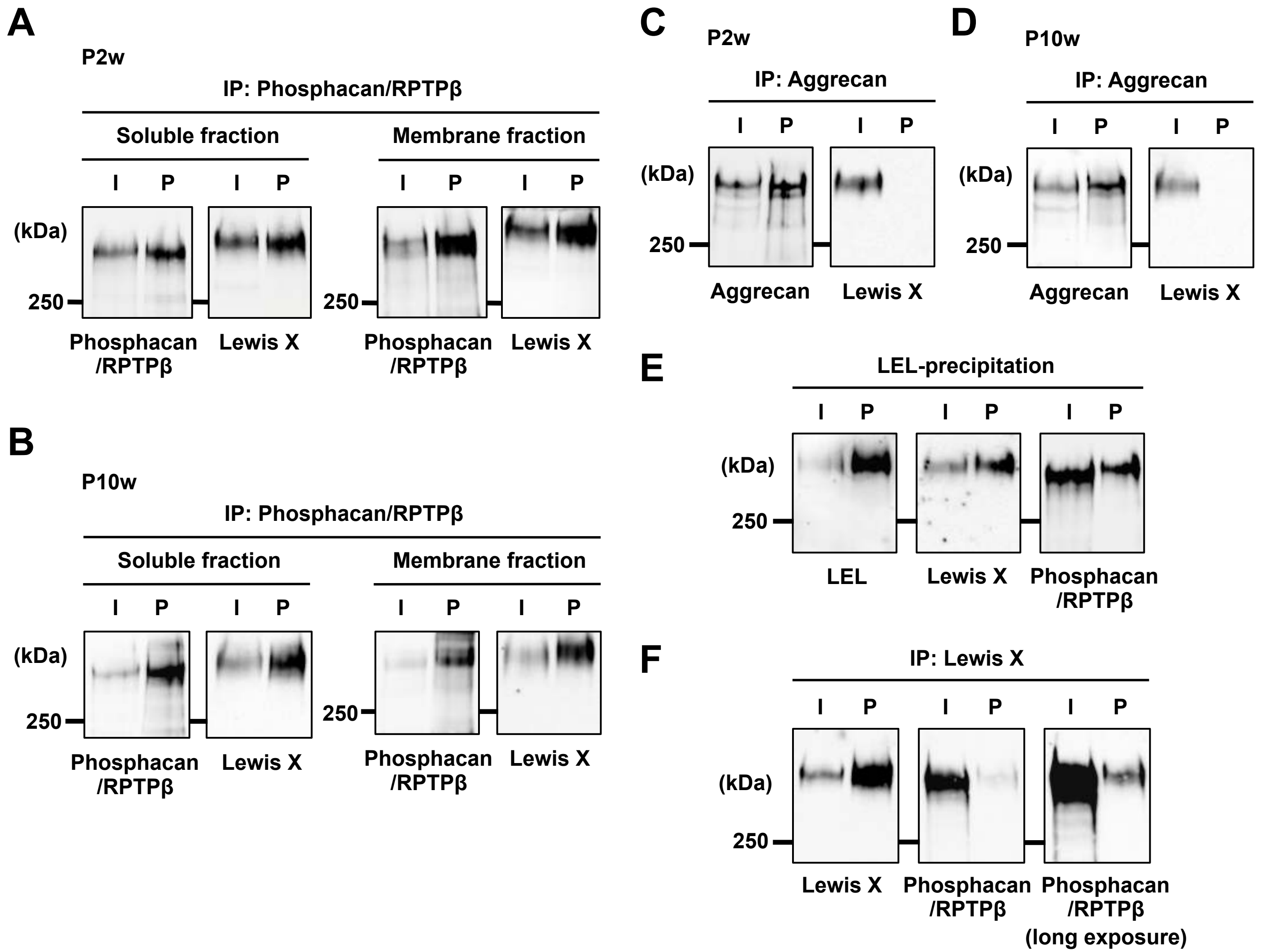

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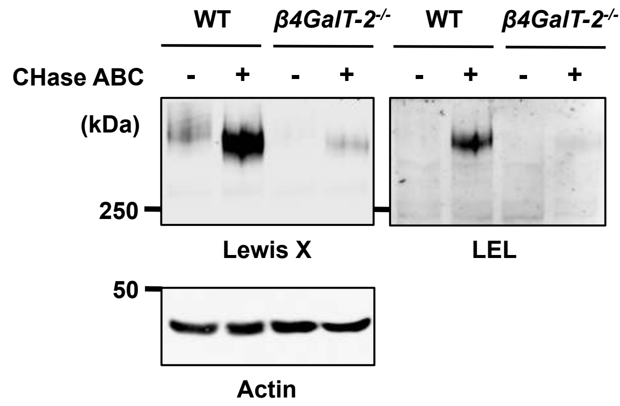
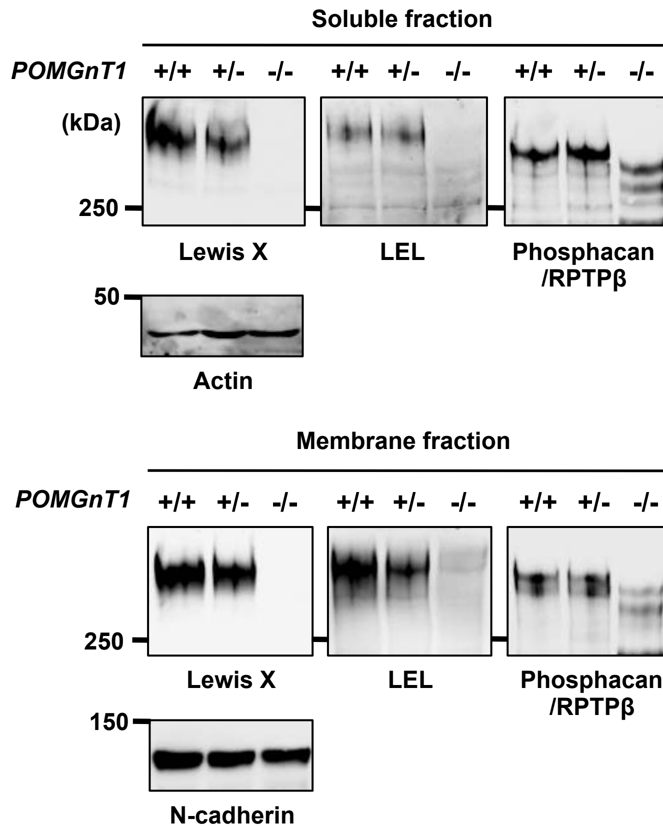
Figure 4





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Supplemental Figure S1

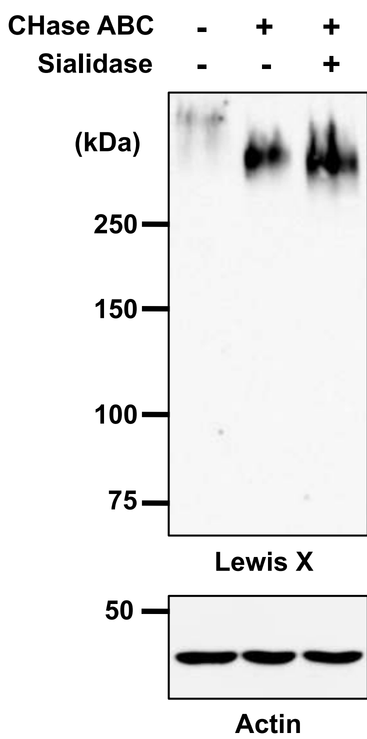

Figure S1. **The Lewis X immunoreactivity after the sialidase treatment.**
The soluble fraction from the P2w brain was successively digested with CHase ABC and sialidase. Digested samples were immunoblotted with the anti-Lewis X mAb. Anti-actin was used as a loading control. In the Lewis X immunoblot, the sialidase treatment resulted in a slight downward shift of the molecular weight of the band over 250 kDa, which indicated a successful sialidase digestion. Note that the sialidase treatment slightly enhanced the Lewis X immunoreactivity of the band over 250 kDa, but did not raise additional bands.