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ORIGINAL ARTICLE

Molecular characterization and expression analyses of ST8Sia II and IV in piglets during postnatal development: lack of correlation between transcription and posttranslational levels

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Abstract The two mammalian α2,8-polysialyltransferases (polyST's), ST8Sia II (STX) and ST8Sia IV (PST), catalyze synthesis of the α2-8-linked polysialic acid (polySia) glycans on neural cell adhesion molecules (NCAMs). The objective of this study was to clone the coding sequence of the piglet ST8Sia II and determine the mRNA expression levels of ST8Sia II, ST8Sia IV, NCAM and neuropilin-2 (NRP-2), also a carrier protein of polySia, during postnatal development. The amino acid sequence deduced from the coding sequence of ST8Sia II was compared with seven other mammalian species. Piglet ST8Sia II was highly conserved and shared 67.8 % sequence identity with ST8Sia IV. Genes coding for ST8Sia II and IV were differentially expressed and distinctly different in neural and non-neural tissues at postnatal days 3 and 38. Unexpectedly, the cellular levels of mRNA coding for ST8Sia II and IV showed no correlation with the posttranslational level of polySia glycans in different tissues. In contrast, mRNA abundance coding for NCAM and neuropilin-2 correlated with expression of ST8Sia II and IV. These findings show that the cellular abundance of ST8Sia II and IV in postnatal piglets is regulated at the level of translation/posttranslation, and not at the level of transcription, a finding that has not been previously reported. These studies further highlight differences in the molecular mechanisms controlling polysialylation in adult rodents and neonatal piglets.

Keywords α2,8-polysialyltransferases · Polysialic acid · Piglet ST8Sia II & IV · NCAM · Neuropilin-2 · Posttranslation regulation

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Introduction

Sialic acids (Sias) are a family of nine-carbon acidic amino sugar that frequently occupy the non-reducing termini of sialylated glycoconjugates. ST8Sia is a family of di-, tri-, oligo- and polysialyltransferases (ST's) that catalyze the transfer of Sia from its activated sugar nucleotide precursor, CMP-Sia, to terminal galactosyl or sialyl residues on N- and O-linked glycan chains [1–3]. By definition, currently all members of the ST8Sia family catalyze synthesis of the α2,8-ketosidic linkage. To date, six members of the ST8Sia family have been cloned and classified into two sub-groups, ST8Sia I, V, VI, representing mono/disialyltransferases, and ST8Sia II, III, IV, representing tri/oligo- and polysialyltransferases. This classification is based on the similarity of their amino acid sequences, their sialylmotif domains and their substrate specificity, gene structure and structure of their cognate biosynthetic glycans. ST8Sia II and IV are the only two mammalian polysialyltransferases (polyST's) responsible for synthesizing the polySia glycotope [3–5].

The predominate mammalian polySias are linear homopolymers consisting of internally linked α2-8-*N*-acetylneuraminic acid (Neu5Ac; Sia) residues that can extend to chain lengths, or degree of polymerization (DP) greater than 400 Sia residues when accurately analyzed under non-hydrolytic conditions [6]. As such, polySia is a large "space-

filling" cell surface glycoprotein which, by steric effects, is postulated to modulate cell-cell interactions [7]. Interestingly, expression of the polySia glycan on the surface of human NK cells can regulate expression of NCAM and the DP of its polySia glycans according to their activation state, similar to how polySia-NCAM can modulate the nervous system [8]. In mammalian cells, including hematopoietic progenitor cells and human neuroblastomas, the polySia glycans are covalently attached posttranslationally to the major carrier protein, NCAM. This modification regulates many cell-cell adhesive properties of NCAM [9]. In addition to NCAM, polysialylation occurs on a restricted set of other glycoproteins including the α-subunit of the sodium channel in adult rat brain [10], neuropilin-2 (NRP-2) on human dendritic cells [11] and the human milk glycoprotein, CD36 [12]. The synaptic CAM, SynCAM1 (or Cadm1) that is expressed on NG2 glia cells, is also polysialylated and controls SynCAM 1 function [13, 14]. Neuropilin-2 (NRP2) is a co-receptor for class 3 semaphorins and vascular endothelial growth factors involved in axon guidance and angiogenesis [15]. A role for polySia-NCAM in neuronal differentiation and proliferation of adult stem cells from inner ear spiral ganglion neurons has recently been described [16].

As the major carrier of polySia, polysialylated NCAM is implicated in a wide range of biological processes and functions by attenuating the adhesive property of cells [3, 9, 17], which in turn facilitates neural cell migration [18], neurite outgrowth [19] and synaptogenesis [20, 21]. NCAM knockout mice lacking polysialylated NCAM are defective in spatial learning and memory due to impaired development in the hippocampus and olfactory bulb [22, 23]. In embryonic tissues, NCAM is heavily polysialylated, whereas the majority of NCAM in adult tissues lacks extended polySia chains except in regions of the brain, eyes and olfactory system associated with neural plasticity [20, 21, 24–28]. The newest function to emerge for polySia is its key role as a "reservoir" for neutrophic factors and neurotransmitters. The first experimental evidence for the "reservoir" function of polySia was described by Kanato, Kitajima and Sato (2008) their studies provided direct biochemical evidence that polySia can bind to a number of "bio-active" neurotrophins and growth factors to regulate neural function" [29]. This new development, has been advanced recently by Sato and Kitajima and colleagues who have shown that polySia can bind to a number of "bioactive" neurotrophins and growth factors to regulate neural function. These include brain derived neurotrophic factor (BDNF), neurotrophin-2, nerve growth factor (NGF) [29–31]; fibroblast growth factor 2 (FGF-2) [32] and neurotransmitters, including dopamine, norepinephrine and epinephrine [33]. The binding complex between polySia and BDNF is very large, exceeding 2500 kDa, and is postulated to sequester and thus extend the effect of neurotrophin function, as recently reviewed [34].

It is well established that the level of polySia on NCAM decreases rapidly after birth. In rodents, the level of polySia decreases by ~70 % during the second and third week of postnatal development leading to NCAM's with reduced levels of polySia [35]. In contrast to the extended polySia chains on NCAM, gangliosides contain shorter glycans of α2-8-linked di- and triSia residues. These common structural features also function in cell-cell adhesive processes [36–38], cell differentiation [39] and signal transduction pathways [40, 41]. Mammalian ST8Sia II and IV are homologous proteins with 59 % amino acid sequence identity [42, 43]. Studies in mice showed that ST8Sia II is principally responsible for polysialylation of NCAM, primarily in the embryonic and early postnatal periods, whereas ST8Sia IV is the predominant polyST expressed in adult brain [35]. ST8Sia IV is reported to polysialylate neuropilin-2 [15] and to synthesize longer polySia chains than ST8Sia II [44]. ST8Sia II and IV have been postulated to show a synergistic effect on synthesis of longer polySia chains on NCAM [45]. The coding sequences for ST8Sia II and IV have been cloned and characterized in several species, including humans and rodents [46, 47].

Although rodents are the most commonly used animal models for biomedical studies, there has been an increased interest in using postnatal piglets as the preferred model because they are genetically closer to humans, share similar physiology and anatomical structures with human infants and have comparable nutritional requirements [48]. Importantly as well, the piglet brain more closely resembles the human brain in anatomic structure and developmental growth patterns [2, 49].

There is a dearth of information on the molecular mechanism underlying how dietary sialylated glycoconjugates can enhance cognitive function and memory [2, 50]. Accordingly, we initiated studies to clone, sequence and characterize the coding region of ST8Sia II in the postnatal piglet. The gene expression profiles for ST8Sia II, IV, and their cognate glycan, polySia, was examined. We also quantified and correlated the expression profiles for NCAM and neuropilin-2 proteins and then systematically analyzed expression of ST8Sia II, IV, polySia, NCAM and neuropilin-2 in neural and non-neural tissues during development in postnatal piglets at 3 and 38 days of age.

Experimental procedures

Animals and tissue collection Three days old male domestic piglets (*Sus scrofa*, Landrace/Large White cross) weighing 1.5–2.4 kg were purchased from a commercial piggery in Xiamen City, China. The piglets were fed a standard sow milk replacer diet containing a mixture of soy/whey/casein (50:38:12) proteins until day 38 when they were euthanized by an intravenous injection of sodium pentothal (50 mg/kg),

as previously described [50]. Tissues from the brain prefrontal cortex, hippocampus, liver, spleen, heart, pancreas, skeletal muscle and kidney were collected after dissection and stored in RNA Safer II ® solution (Omega Bio-tek, China, Cat. No: R0424-02) for qPCR experiments, and at −80 °C for subsequent Western blot analyses. The study protocols were approved by the Animal Ethics Committee at Xiamen University.

Molecular characterization of ST8Sia II For the molecular characterization of ST8Sia II, total RNA was extracted from the hippocampus, prefrontal cortex and non-neural tissues as described above, and analyzed by quantitative real-time PCR using the RNeasy Lipid Tissue Mini Kit (Qiagen, China, Cat. No: 74804) [24, 35] and the RNeasy Mini Kit (Qiagen, China, Cat.No.74104) [51]. The cDNA was prepared by reverse transcription (RT) using the RevertAid First Strand cDNA Synthesis Kit (Fementas, China Cat.No: K1621), in reaction mixtures containing 5 μg total RNA, 0.5 μl oligo (dT)18 primer and 0.5 μl of a random hexamer primer, 4 μl 5X reaction buffer, 1 μl RiboLock™ RNase Inhibitor (20 u/μl), 2 μl 10 mM dNTP Mixture, 1 μl RevertAid™ M-MuLV Reverse Transcriptase (200 u/μl), and nuclease-free water to 20 μl. The RT reaction mixtures were incubated for 5 min, 25 °C, followed by an additional 60 min incubation at 42°. The reaction was terminate by heating at 70 °C for 5 min. Approximate 1 μg of cDNA was used as the template for PCR amplification using the Advantage® 2 PCR Kit (Clonetech, Beijing; Cat. No: 639207). PCR primers were designed with the reference from Human ST8Sia II (GenBank: NM_006011) and a predicted pig ST8Sia II complementary DNA (cDNA) sequence (GeneBank: XM_001924887). For cloning, the following two pairs of primers were designed: 5′ ATGCAGCTGCAGTTCCGGAGCTGGA3′ and 5′ GGAGGTGTAGCCATACTTGAGG3′; 5′ ATCTTCGACCGAGACAGCAC3′ and 5′ TTACGTGGCCCCATCGCA 3′. PCR was carried out by an initial heating (95 °C, 5 min) followed by 40 cycles of amplification at 95 °C. Samples were then denatured for 1 min, 60 °C and annealed for 1 min, 72 °C. This was extended for 1 min after the 40 cycles of amplification and then increased to 5 min, at 72 °C. The PCR products were purified by electrophoresis on agarose gels (Invitrogen, China), and the single DNA band isolated using the TIANgel Midi Purification Kit, Tiangen, China, (Cat.No: DP209). This DNA was inserted into the pMD18-T vector (Takara, China; Cat. No: D101A) and transformed into *E. coli* DH5α. Five positive colonies of each band were selected to send to Sangon (Shanghai, China) for bidirectional sequencing of the 18S rRNA gene using universal M13 sequencing primers for DNA sequencing. The MAFFT version 6 program [52] was used to compare the amino acid sequence of ST8Sia II with the porcine ST8Sia IV, and the seven other species and to generate the phylogenetic tree.

Quantitative real-time PCR analysis Expression levels of mRNA for the piglet ST8Sia II and the reference genes, Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) and the TATA-Box-Binding Protein (TBP) [53] were quantified using the SDS7300 real time PCR system (Applied Biosystems, China). Primer sequences and accession numbers for HPRT1, TBP, ST8Sia II & IV and neuropilin-2 genes are described in Table 1. Each qPCR reaction mixture (20 μl) contained the following: 10 μl of 1X FastStart Universal SYBR Green Master Mix (Roche, China, Cat. No: 04913914001), 50 ng cDNA and 300 nM primers for each gene, and double distilled water to 20 μl. All samples were analyzed in duplicate. Relative mRNA levels were determined from the equation: $(E(\text{target}))^{\Delta Ct,(\text{calibrator-test})}/(E(\text{ref}))^{\Delta Ct,(\text{calibrator-test})}$, where E(target) and E(ref) are the PCR efficiencies for the target and reference genes respectively, and Ct is the threshold cycle number. For the reference genes, a normalization factor was obtained using the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/), which is an arithmetic based on a Microsoft Excel method to confirm the most stable reference genes [54]. From these analyses, a gene expression normalization factor was calculated for each sample, based on the geometric mean of a user-defined number of housekeeping genes [54]. Relative expression levels of individual target genes were obtained using the normalization factor obtained with the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/).

Western blot analysis — For Western blot analyses, ~100 mg of tissue were homogenized in 1 ml of cold lysis buffer (50 mM HEPES, pH7.5, 2 mM EDTA, 50 mM NaCl, 2 % TritonX-100, 1 mM DTT, and 1 mM PMSF in a pre-chilled glass homogenizer with ~15 strokes. After cell disruption, the homogenate was transferred to a 2 ml Eppendorf tube and placed in ice for 1 h before centrifugation at 4 °C, 10 min at 20,000 x g. Protein levels in the supernatant fractions were determined using the BCA Protein Assay Kit (23227, Pierce). For analysis of the NCAM protein, all samples were treated with neuraminidase (1U/ml, N2133, Sigma-Aldrich) at 37 °C for 3 h. This pretreatment step was not carried out when determining the expression level of polySia-NCAM. Fifty μg of protein for each sample was subjected to SDS-PAGE electrophoresis (5 % stacking and 7 % running gel) before electrotransfer to a PVDF membrane. The membranes were first blocked with 5 % skim milk for 1 h, then incubated at 4 °C overnight with an anti-polySia primary antibodies specific for detecting polySia-NCAM (mAb5324, Millipore Corp.) at 1:1000 dilution or an anti-NCAM mAb (H00004684-M01, Abnova) at 2.5 μg/mL. After washing the PVDF membranes with TBST (10 mM Tris, 150 mM NaCl, 0.05 % Tween-20)

Table 1 Primer sequences and accession number used in real-time PCR

Gene	Primer sequences	Products size(bp)	Accession number
HPRT1	F:5′-ACCACACCACTATTGAATG-3′ R:5′-TGAAACTGACTGCTTACTTT-3′	178	NM_001032376
TBP	F:5′-GTTATGAGCCAGAGTTGTT-3′ R:5′- CTTTAGGATAGGGTAGATGTTC -3′	155	DQ178129
ST8SiaII	F:5′-GAGGATACTGGCTGACTA-3′ R:5′-ATACTTGAGGCTGTCGTA-3′	173	JQ740150
ST8SiaIV	F:5′-CGATAAGTCTGCTCCTGAT-3′ R:5′-AGAATGCTGGAAGATTGAAG-3′	167	NM_001037322
Neuropilin-2	F:5′-TTGCCATTGATGACATTCG-3′ R:5′-AGAAGAGTTGCTCCAGTC-3′	161	GACC01000014

three times, the membranes were incubated with a secondary antibody (goat-anti-mouse lgG, BA1050, BOSTER) for 1 h at room temperature. After a second washing cycle with TBST, the immune complexes were detected by enhanced chemiluminescence using CW0049A for polySia (Beijing CWBio Co., Ltd., China) and RPN2235 for NCAMs, (GE Healthcare, USA). Membranes were then exposed to X-ray film. Quantification of the protein bands was carried out by densitometry under non-saturating conditions. Band density was analyzed using the Quantity One software (Bio-Rad).

Immunofluorescence - Consecutive coronal sections of 20 μm (brain) and 5 μm thickness from the piglet's hippocampus, frontal cortex, spleen and kidney were prepared using a freezing microtome (LEICA CM 1950, Germany). Brain sections were fixed in ice-cold 4 % paraformaldehyde for 15 min, rinsed in phosphate-buffered saline (PBS), and permeabilized with 0.3 % Triton X-100 for 30 min, followed by blocking with 0.3 % goat serum in PBS (Rm. Temp). The thin sections were incubated overnight (4 °C) with a primary antibody specific for detecting the polySia moiety of polySia-NCAM (1:200; MAB5324, Millipore, USA). After washing with PBS, sections were incubated with Alexa Fluor 488-conjugated secondary antibody (1:200; 115-545-075, Jackson ImmunoResearch, USA) for 1 h. at room temperature. All sections were counterstained with DAPI (H-1200, VECTOR laboratories, USA). Images were analyzed using a confocal microscope (FV1000, Olympus, Japan) with mage Pro Plus 6.0 software.

Results

Molecular characterization of porcine ST8Sia II ST8Sia II has been cloned and studied in several mammalian species, including human and rodents, but not in the piglet. To further our understanding of the molecular mechanisms underlying the role of sialylated glycoconjugates in cognition and memory [2], we deemed it important to clone and characterize the ST8Sia II gene from piglets, a study that has not been previously reported. To this end, with the reference gene from human ST8Sia II (GeneBank: NM_006011) and a predicted pig ST8Sia II cDNA sequence (GeneBank: XM_001924887), two pairs of primers were designed for cloning ST8Sia II by PCR. A coding sequence with a length of 1128 bp that encoded 375 amino acids was obtained and characterized (Fig. 1). No nucleotide variants were identified among three piglets. The coding region of the piglet ST8Sia II cDNA showed high homology to the human in both nucleotide and protein sequences. There were 77 nt differences with 93 % identity compared to the human counterpart (NM_006011) (Fig. 2). The open reading frame coded for 375 amino acids with the nucleotide start ATG (methionine) and ended with TAA as the stop codon. Of the 375 amino acids encoded by this sequence, only seven, labeled with the asterisk (*) in Fig. 2a, were found to differ between human and piglet. These amino acid differences included alanine and glutamic acid in the pig sequence replacing serine and glutamine in the human protein sequence at position 84 and 173, respectively. Further, threonine replaced alanine at position 177, isoleucine replaced leucine at position 198, asparagine replaced lysine at position 308, aspartic acid replaced glutamic acid at position 360 and arginine replaced glutamine at position 370 (Fig. 2a).

As noted, the family of sialyltransferases consist of six members designated ST8Sia I-VI. As shown in Fig. 1, the piglet ST8Sia II is similar to other members of the ST8Sia family, and shares common structural features including a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region, and catalytic domains containing the highly conserved sialyl motifs (SM). These motifs include SM L (Long), SM S (Short), and SM VS (Very Short). The novel polybasic polyST domain, designated PSTD, which is unique to the two mammalian polysialyltransferases, is also present in the piglet ST8Sia II (Fig. 1). This polymerization-related domain of 32 amino acids is immediately upstream of SM S, and is essential for polysialylation (4, 55).

The ortholog protein sequences for ST8Sia II were retrieved for seven other species including human (*Homo sapiens*, NM_006011), mouse (*Mus musculus*, NM_009181), rat (*Rattus norvegicus*, NM_057156), ox (*Bos*

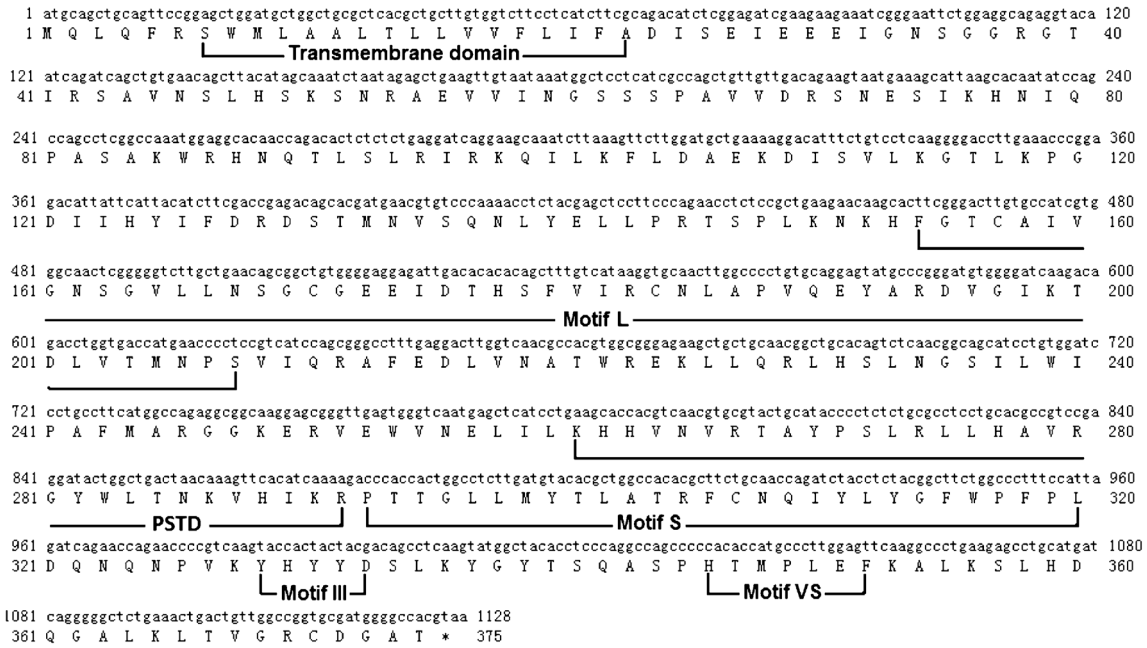

Fig. 1 Sequence and structural features of piglet ST8Sia II. The 1128 nucleotide and 375 amino acid sequences of the piglet ST8Sia II are shown in the upper and lower panel, respectively. The classical structural features, including the transmembrane domain and the catalytic domains containing the highly conserved sialyl motifs (SM) L (Long), S (Short), III VS (Very Short) and the polybasic polysialyltransferase domain (PSTD), are indicated in bold

taurus, NM_001098113), zebrafish (*Danio rerio*, NM_153662), dog (*Canis lupus familiaris*, NM_001097548) and chicken (*Gallus gallus*, NM_001001604). The multiple amino acid sequence alignment for these species showed that secondary to bovine (99 %), the amino acid consensus sequence between piglet and human was 98 %, which is a closer relationship than any of the other species (Fig. 2a). As shown in Fig. 2b, a phylogenetic tree for these species was generated to determine the genetic distance between the ST8Sia II genes. The values for the bar shown in Fig. 2b represent nucleotide diversity. These results show that the piglet shares a remarkably closer genetic relationship to the human ortholog than the mouse, rat, dog, chicken and zebrafish. It is well established that ST8Sia II and IV can both independently catalyze synthesis of the polySia glycan on NCAM, and that their protein sequences are highly similar [42, 43]. As shown in Fig. 3, our sequence alignment showed that the piglet ST8Sia II and IV share 67.8 % identity in amino acid sequence, which is comparable to that in human and mouse.

Gene expression profiles of ST8Sia II and ST8Sia IV during postnatal development in the piglet While ST8Sia II and IV belong to the same subfamily of sialyltransferases genes, there is little information on the expression profile for these genes during postnatal development in the piglet. To better understand this relationship, we carried out studies to characterize the developmental expression profile for these genes in different neural and non-neural tissues from piglets at postnatal day 3 and 38. This period of postnatal development is approximately equivalent to 15 day-old and 10 month old humans, respectively [49]. Tissue from the central nervous system (CNS), including the hippocampus and prefrontal cortex, and non-CNS tissues, including heart, skeletal muscle, kidney, spleen, pancreas and liver were isolated and analyzed by quantitative real-time PCR as described under "Experimental Procedures". In partial agreement with earlier studies in rodents [24], expression of genes encoding for ST8Sia II (Fig. 4a) and ST8Sia IV (Fig. 4b) in the piglet were expressed at lower levels in the hippocampus and frontal cortex than in non-CNS tissues. With the exception of muscle and pancreas, the mRNA levels for ST8Sia II in hippocampus, prefrontal cortex, heart, liver, spleen and kidney showed a significant decrease during postnatal development from day 3 to 38 (Fig. 4a). Similar to ST8Sia II, the mRNA level for ST8Sia IV in most tissues was higher at day 3, compared to day 38, particularly in the hippocampus, prefrontal cortex, heart, spleen and kidney, the exception being liver, pancreas and muscles (Fig. 4b). This differential level of gene expression for ST8Sia II and IV in piglets is similar to that reported in mouse brain [35]. During postnatal development, the abundance level of message for ST8Sia IV decreased at a slower rate than ST8Sia II (Fig. 4b), a finding also resembling that found in mouse brain (35).

Expression levels of the cognate polySia glycans in postnatal piglet tissues — Expression levels for the cognate polySia glycans synthesized in the hippocampus, frontal

a

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1          11         21         31         41                  1          11         21         31         41
MQLQFRSWML AALTLLVVFL IFADISEIEE EIGNSGGRGT IRSAVNSLHS   50    ARDVGLKTDL VTMNPSVIQR AFEDLVNATW REKLLQRLHS LNGSILWIPA  242 Human
MQLQFRSWML AALTLLVVFL IFADISEIEE EIGNSGGRGT IRSAVNSLHS   50    ARDVGIKTDL VTMNPSVIQR AFEDLVNATW REKLLQRLHS LNGSILWIPA  242 Pig
MQLQFRSWML AALTLLVVFL IFAD------ ---NSGGRGT IRSAVNSLHS   41    ARDVGLKTDL VTMNPSVVQR AFEDLVNATW REKLLQRLHS LNGSILWIPA  233 Ox
MQLQFRSWML AALTLLVVFL IFADISEIEE EIGNSGGRGT IRSAVNSLHS   50    ARDVGLKTDL VTMNPSVIQR AFEDLVNATW REKLLQRLHG LNGSILWIPA  242 Mouse
MQLQFRSWML AALTLLVVFL IFADISEIEE EIGNSGGRGT IRSAVNSLHS   50    ARDVGLKTDL VTMNPSVIQR AFEDLVNATW REKLLQRLHG LNGSILWIPA  242 Rat
MQLQFRSWML AALTLLVVFL IFADISEIEE EIGDSGGRGT IRSAVNSLHS   50    GRDVGLKTDL VTMNPSVIQR AFEDLVNATW REKLLQRLRS LNGSILWIPA  199 Dog
MPLPCRGWTL ALLTLLVGFL IFADISEIEG ENGSSGGRGT IRSAVNSLHS   50    SQDVGTKTDL VTMNPSVIQR AFEDLMNETW REKLLQRLHS LNGSILWIPA  242 Chicken
MSFEFRILMF GIGTALVIFV IIADISEVEE EIANI--EDS RKFHLKSVAL   48    AADVGLRTSL VTMNPSVVQR AFQDLNSEEW VQRFVQRPQS LSGSVLWIPA  248 Zebrafish
                                                    *
KSNRAEVVIN GSSSPAVVDR SNES------ --IKHNIQPA SSKWRHNQTL   92    FMARGGKERV EWVNELILKH HVNVRTAYPS LRLLHAVRGY WLTNKVHIKR  292 Human
KSNRAEVVIN GSSSPAVVDR SNES------ --IKHNIQPA SAKWRHNQTL   92    FMARGGKERV EWVNELILKH HVNVRTAYPS LRLLHAVRGY WLTNKVHIKR  292 Pig
KSNRAEVVIN GSSSPAVVDR SNES------ --IKHNIQPA SAKWRHNQTL   83    FMARGGKQRV EWVNELILKH HVNVRTAYPS LRLLHAVRGY WLTNKVHIKR  283 Ox
KSNRAEVVIN GSSPPAVADR SNES------ --LKHNIQPA SSKWRHNQTL   92    FMARGGKERV EWVNALILKH HVNVRTAYPS LRLLHAVRGY WLTNKVHIKR  292 Mouse
KSNRAEVVIN GSSLPAVADR SNES------ --LKHSIQPA SSKWRHNQTL   92    FMARGGKERV EWVNALILKH HVNVRTAYPS LRLLHAVRGY WLTNKVHIKR  292 Rat
KSN------- ---------- ---------- ---------- ----------   53    FMARGGKERV EWVNELILKH HVNVRTAYPS LRLLHAVRGY WLTNKVHIKR  249 Dog
KSNRAEVVIN DSSSPAVVDR SNES------ --IKHNIKPA SSKWRHNQTL   92    FMAKGGKERV EWVNELILKH RINVRTAYPS LRLLHAVRGY WLTNKVHIKR  292 Chicken
QSNRSSDLNA APTSLVTYRK SKVSSLASPS DIKRKTSNSS SSEWTFNRTL   98    FMAKGGEERV EWAIRLILLH TVNVRTAFPS LRLLHAVRGY WLTNHVQIKR  298 Zebrafish
                                                              *
SLRIRKQILK FLDAEKDISV LKGTLKPGDI IHYIFDRDST MNVSQNLYEL  142    PTTGLLMYTL ATRFCKQIYL YGFWPFPLDQ NQNPVKYHYY DSLKYGYTSQ  342 Human
SLRIRKQILK FLDAEKDISV LKGTLKPGDI IHYIFDRDST MNVSQNLYEL  142    PTTGLLMYTL ATRFCNQIYL YGFWPFPLDQ NQNPVKYHYY DSLKYGYTSQ  342 Pig
SLRIRKQILK FLDAEKDISV LKGTLKPGDI IHYIFDRDST MNVSQNLYEL  133    PTTGLLMYTL ATRFCNQIYL YGFWPFPLDQ NQNPVKYHYY DSLKYGYTSQ  333 Ox
SLRIRKQILK FLDAEKDISV LKGTLKPGDI IHYIFDRDST MNVSQNLYEL  142    PTTGLLMYTL ATRFCNQIYL YGFWPFPLDQ NQNPVKYHYY DSLKYGYTSQ  342 Mouse
SLRIRKQILK FLDAEKDISV LKGTLKPGDI IHYIFDRDST MNVSQNLYEL  142    PTTGLLMYTL ATRFCNQIYL YGFWPFPLDQ NQNPVKYHYY DSLKYGYTSQ  342 Rat
----RKQILK FLDAEKDISV LKGTLKPGDI IHYIFDRDST MNVSQNLYDL   99    PTTGLLMYTL ATRFCNQIYL YGFWPFPLDQ NQNPVKYHYY DSLKYGYTSQ  299 Dog
SLKIRKQILK FLDAEKDISV LKGTLKPGDV IHYVFDRDST MNVSQNLYEL  142    PTTGLLMYTL ATRFCNRIHL YGFWPFPLDQ NQQPVKYHYY DSLKYGYTSQ  342 Chicken
SNLIRKNILK FLDAERDISI LKSTFKPGDV IHYIFDRQST TNISENLYHL  148    PTTGLLMYTM ATRFCDEIHL YGFWPFAHDP DGKPVKYHYY DTLTYHYTSS  348 Zebrafish
                                 * *                                        *          *
LPRTSPLKNK HFGTCAIVGN SGVLLNSGCG QEIDAHSFVI RCNLAPVQEY  192    ASPHTMPLEF KALKSLHEQG ALKLTVGQCD GATKKKK               379 Human
LPRTSPLKNK HFGTCAIVGN SGVLLNSGCG EEIDTHSFVI RCNLAPVQEY  192    ASPHTMPLEF KALKSLHDQG ALKLTVGRCD GATKKKK               379 Pig
LPRTSPLKNK HFGTCAIVGN SGVLLNSGCG QEIDTHSFVI RCNLAPVQEY  183    ASPHTMPLEF KALKSLHEQG ALKLTVGQCD GATKKKK               370 Ox
LPRTSPLKNK HFQTCAIVGN SGVLLNSGCG QEIDTHSFVI RCNLAPVQEY  192    ASPHTMPLEF KALKSLHEQG ALKLTVGQCD GATKKKK               379 Mouse
LPRTSPLKNK HFQTCAIVGN SGVLLNSGCG QEIDTHSFVI RCNLAPVQEY  192    ASPHTMPLEF KALKSLHEQG ALKLTVGQCD GATKKKK               379 Rat
LPRTSPLKNK HFRTCAIVGN SGVLLNSGCG QEIDTHSFVI RCNLAPVQEY  149    ASPHTMPLEF KALKSLHEQG ALKLTVGQCD GATKKKK               336 Dog
LPRTSPLKGK QFPSCAIVGN SGVLLGSGCG PEIDTHSFVI RCNLAPVQEY  192    ASPHTMPLEF KALKTLHQQG ALKLTVGECE GDTKKKK               379 Chicken
LPTVSPMKNQ HYRKCAIVGN SGILLNSSCG REIDSHDFVI RCNLAPVEEY  198    ASPHTMPLEF RTLSALHRQG ALRLHTGPCK PPTKKKK               385 Zebrafish
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b

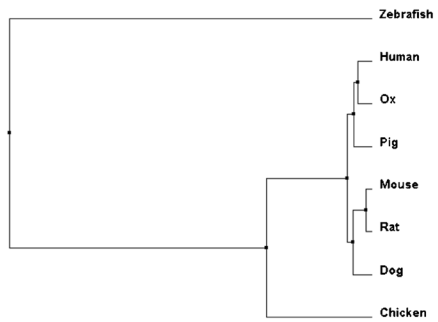

Fig. 2 Genetic conservation in the amino acid sequence of piglet ST8Sia II. **a** Comparison of the alignments of the amino acid sequence of ST8Sia II from human, piglet, mouse, rat, ox, zebrafish, dog and chicken were carried out using the MAFFT version 6 program. These correspond to amino acid numbers 375 in the sequence. The seven amino acids that are different between human and piglet are labeled with an asterisk l (*) above the sequence. **b** The phylogenetic tree for ST8Sia II from the above eight species was generated using a multiple sequence alignment program for the Unix-like operating system (MAFFT version 6). The genetic distance for ST8Sia II was calculated based on the software program as described [52] (http://align.bmr.kyushu-u.ac.jp/mafft/software/)

cortex and non-CNS tissues during postnatal development at days 3 and 38 were determined. Spleen and kidney, both tissues which showed relatively high levels of mRNA for ST8Sia II and IV (Fig. 4a & b), were selected as non-neural tissues to be examined by Western blot analysis using an antibody specific for detecting polySia. As shown in Fig. 5a, higher levels of polySia were synthesized in the hippocampus and prefrontal cortex at day 3 compared with day 38, showing again a developmentally related decrease in polySia during postnatal piglet development. In spite of the high level of mRNA expression for both ST8Sia II and IV (Fig. 4a & b), there was essentially no polySia detected in spleen or kidney at either 3 or 38 days (Fig. 5a). This unexpected finding was confirmed in three individual piglets (Fig. 5a). Immunohistochemistry studies confirmed that polySia was expressed at higher levels in the hippocampus and frontal cortex and showed a decreased expression at day 3 compared with 38 day-old piglets (Fig. 5b). In kidney and spleen, however, where message level for ST8Sia II was high at day 3 and 38 (Fig. 4), expression of the cognate polySia was very low and no polySia was detected in kidney or spleen at 38 days (Fig. 5c and d). These results are also in accord with our Western blot analysis showing no polySia expression in kidney and spleen at day 3 or 38 (Fig. 5a).

Previous studies have reported a correlation between expression level of polySia mRNA and the amount of polySia expressed in mouse brain [35]. As noted above, however, our results differ and show no direct correlation between the

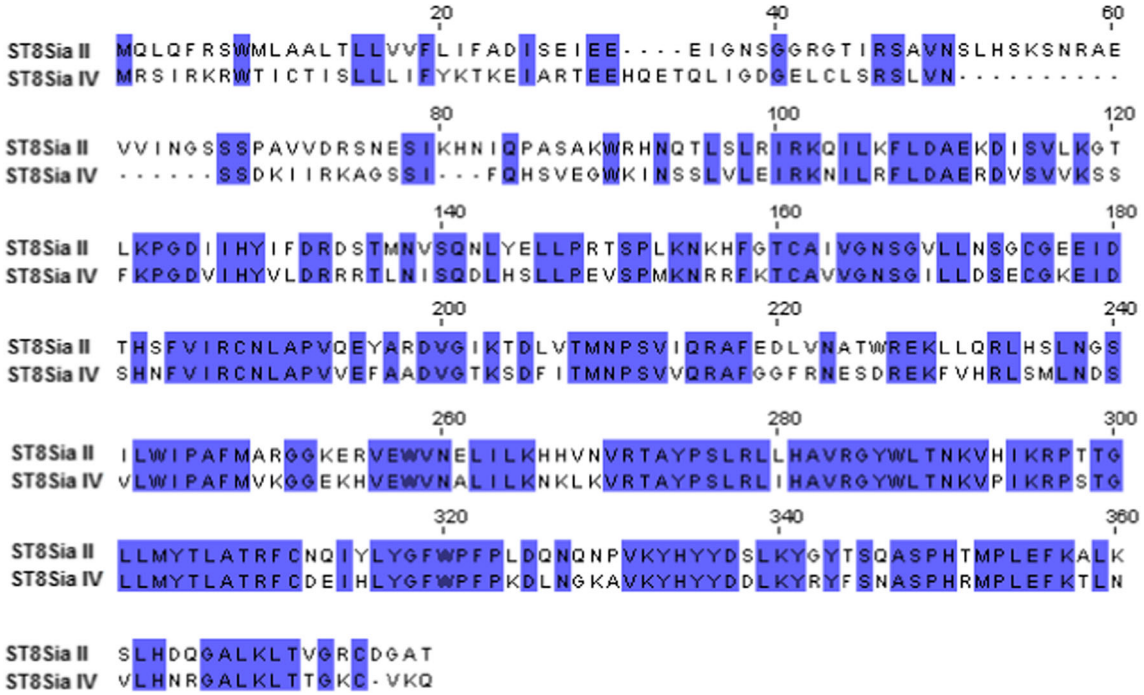

Fig. 3 Comparison of the amino acid sequence similarity between piglet ST8Sia II and ST8Sia IV. The sequence alignment was carried out using the MAFFT version 6 program. Identity in the amino acid consensus sequences between the two polysialyltransferases is indicated by the shaded areas

relatively low mRNA abundance levels for ST8Sia II and IV in the hippocampus and prefrontal cortex in piglets at 3 days of age (Fig. 4a, b) with a corresponding high level of expression of the cognate polySia glycans in the piglet (Figs. 5a & 6a). This finding highlights a fundamental difference between mouse and piglet species. Importantly, our new results are further evidence in support of the seminal findings of Selbach and colleagues who showed that there is often no correlation between mRNA (transcription) and protein (translation) abundance levels (56). Accordingly, our new findings provide further evidence that extends the transcription/transcriptional paradigm to now include post-translational glycosylation modifications, in this case polysialylation in the postnatal piglet.

Several possibilities may explain the lack of correlation between the abundance level of message for ST8Sia II and IV and the abundance level of the cognate posttranslation product, polySia. These include differences in the stability of mRNA for the two transferases, the level of CMP-Sia, the enzymatic activities of the polyST's and/or the abundance level of NCAM expression in different tissues. With respect to the latter possibility, NCAM is the major carrier protein of polySia in brain, and is recognized as a protein substrate for polysialylation catalyzed by both ST8Sia II and IV [26, 55]. An attenuated level or deficiency in NCAM expression levels in different tissues could therefore account for the absence of polySia-NCAM in spleen and kidney (Fig. 5a) even though high levels of message for ST8Sia II/IV are expressed in these tissues (Fig. 4). An inherent instability or half-life of mRNA encoding for ST8Sia II and IV may also be a possible reason for the lack of concordance between message level and abundance of the posttranslational polySia product, suggesting that after synthesis of the mRNA encoding for the polyST's, the message is degraded. Using a quantitative model of genome-scale prediction of synthesis rates of mRNAs and proteins, recent studies by Schwanhausser *et al.* have shown that the cellular abundance of proteins is predominantly controlled at the level of translation and not transcription [56]. This may insure that the cellular abundance of NCAM in brain in early life is regulated such that polySia-NCAM expression remains high during the critical period of postnatal neural development in the piglet. In accord with previous findings that the level of polySia in mouse and embryonic chicken brains declined rapidly after birth [28, 57, 58], the level of polySia expressed in piglet brains also showed a developmentally regulated decline, since it was significantly lower at day 38 than day 3. This highlights for the first time the developmental regulation of polySia expression during development in the brains of postnatal piglets.

Expression levels of NCAM and Neuropilin-2 proteins in neural and non-neural tissues in the postnatal piglets

While NCAM is the major neural glycoprotein that is posttranslationally modified by polySia, other key brain proteins are also polysialylated, including Neuropilin-2 [11, 59], SynCAM 1 [13] and the human milk glycoprotein, CD36

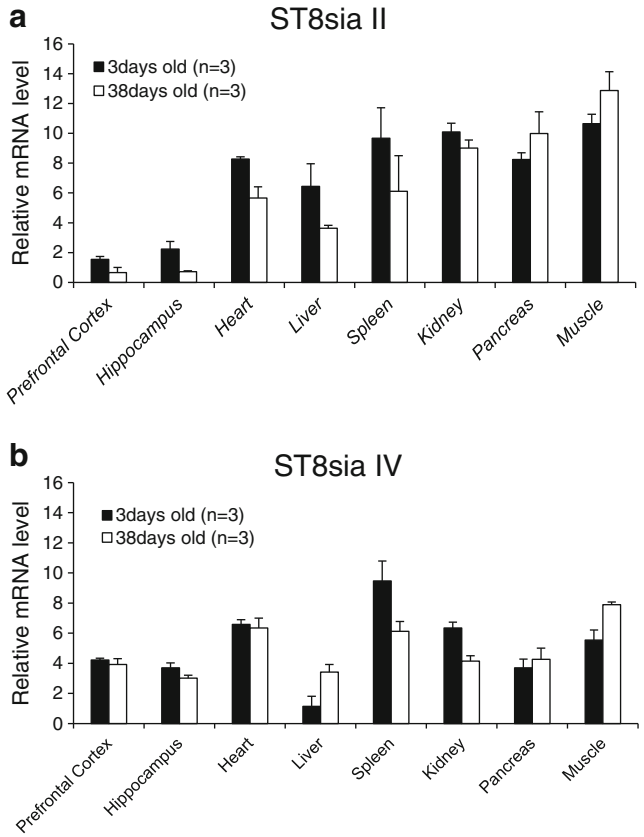

Fig. 4 Comparison of the relative mRNA gene expression levels for the piglet ST8Sia II and IV in different tissues at postnatal days 3 and 38. qRT-PCR was carried out to analyze the level of gene expression for ST8Sia II (panel A), ST8Sia IV (panel B) in the hippocampus, prefrontal cortex, heart, muscle, kidney, spleen, pancreas and liver in postnatal piglets, as described under "Experimental Procedures". HPRT1 and TBP genes were used as the reference genes. The relative level of mRNA for the individual genes was obtained by normalizing to the normalization factor, which was generated from the double reference genes using the geNorm software, as described under "Experimental Procedures". The insert in each panel shows a magnification of the relative message level for ST8Sia II (panel A) and ST8Sia IV (panel B) in the prefrontal cortex and hippocampus

[12]. Functional characterization of the expression levels of the polysialyltransferases that modify these polySia carrier proteins, and the level of the cognate polysialylated proteins themselves, are of critical importance for a better understanding of the molecular mechanisms underlying how expression of these developmentally regulated glycoproteins are controlled. Accordingly, we used an anti-NCAM antibody to determine the expression level of NCAM proteins *via* Western blot analyses in the hippocampus, frontal cortex, spleen and kidney after neuraminidase treatment in postnatal piglets at days 3 and 38. As shown in Fig. 6a, Western blot analysis revealed that the NCAM protein expressed in the hippocampus and prefrontal cortex in three different piglets resided in the ~140 kDa NCAM species, with a minor band at ~120 kDa. There was no expression of NCAM180 in any tissues examined. The 140 kDa NCAM finding was confirmed by quantitative densitometric-analysis, as described under "Experimental Procedures" (Fig. 6b). Surprisingly, given the high level of message expression for ST8Sia II & IV in spleen and kidney (Fig. 4), there was no expression of the ~140 kDa NCAM species in either spleen or kidney at 3 or 38 days (Fig. 6a & b), confirming a loss in expression of NCAM140 in these two tissues. In contrast to the above findings, however, both spleen and kidney expressed NCAM immunoreactive proteins that migrated as an apparent doublet with an apparent molecular mass of ~130 kDa and ~120–110 kDa in 3 but not 38-day old piglets. While the nature of these anti-NCAM reactive proteins remains uncertain, these results support earlier findings that the NCAM ~120–130 kDa species may not be polysialylated [14], and that expression of these proteins undergoes a loss during postnatal development.

Oltmann-Norden *et al.* reported that NCAM-140 and NCAM-180 were the major NCAM isoforms in the brains of newborn rodents. The apparent lack of the 180 kDa NCAM in piglet brains shows that the posttranslational modification of NCAM by polySia is highly tissue specific, cell-type-specific and species-specific, underscoring again the distinct differences between the mechanisms of polysialylation in adult rodents and postnatal piglets. Because there was no confirmed NCAM gene sequences available for piglets during the course of this study, no mRNA level of NCAM were included.

Neuropilin-2, a polysialylated neurotrophin, functions as a co-receptor for class 3 semaphorins, several vascular endothelial growth factors (VEGFs), and is crucial for repulsive axon guidance, vascularization, and angiogenesis [15, 60, 61]. In contrast to NCAM, there is no information regarding polysialylation of neuropilin-2 in piglets. To provide greater insight into this understudied problem, we examined the mRNA expression levels for neuropilin-2 in neural and non-neural tissues during postnatal development in the piglet. Message expression for neuropilin-2 in piglets is developmentally regulated, as shown by the substantially lower level of expression at 38 days compared with 3 days in the CNS and most non-CNS tissues, except muscle (Fig. 7). In the CNS tissue, neuropilin-2 expression was higher in the hippocampus than the prefrontal cortex in both 3 and 38-day old piglets. The level of mRNA expression for neuropilin-2 was highest in kidney, hippocampus and spleen at postnatal day 3 and only in muscle at postnatal day 38 (Fig. 7). Thus, similar to expression of both message and protein levels coding for polySia and NCAM, gene expression of neuropilin-2 is developmental regulated and tissue specific. Interestingly, the mRNA expression level for neuropilin-2 correlated with expression of both ST8Sia II and ST8Sia IV ($P<0.01$, Pearson Correlation Analysis). While this result is in accord with recent findings that polysialylation of neuropilin-2 is catalyzed only by ST8Sia IV [15], we found that the piglet ST8Sia II can also catalyze polysialylation of neuropilin-2. This finding highlights again differences between rodent and postnatal piglet

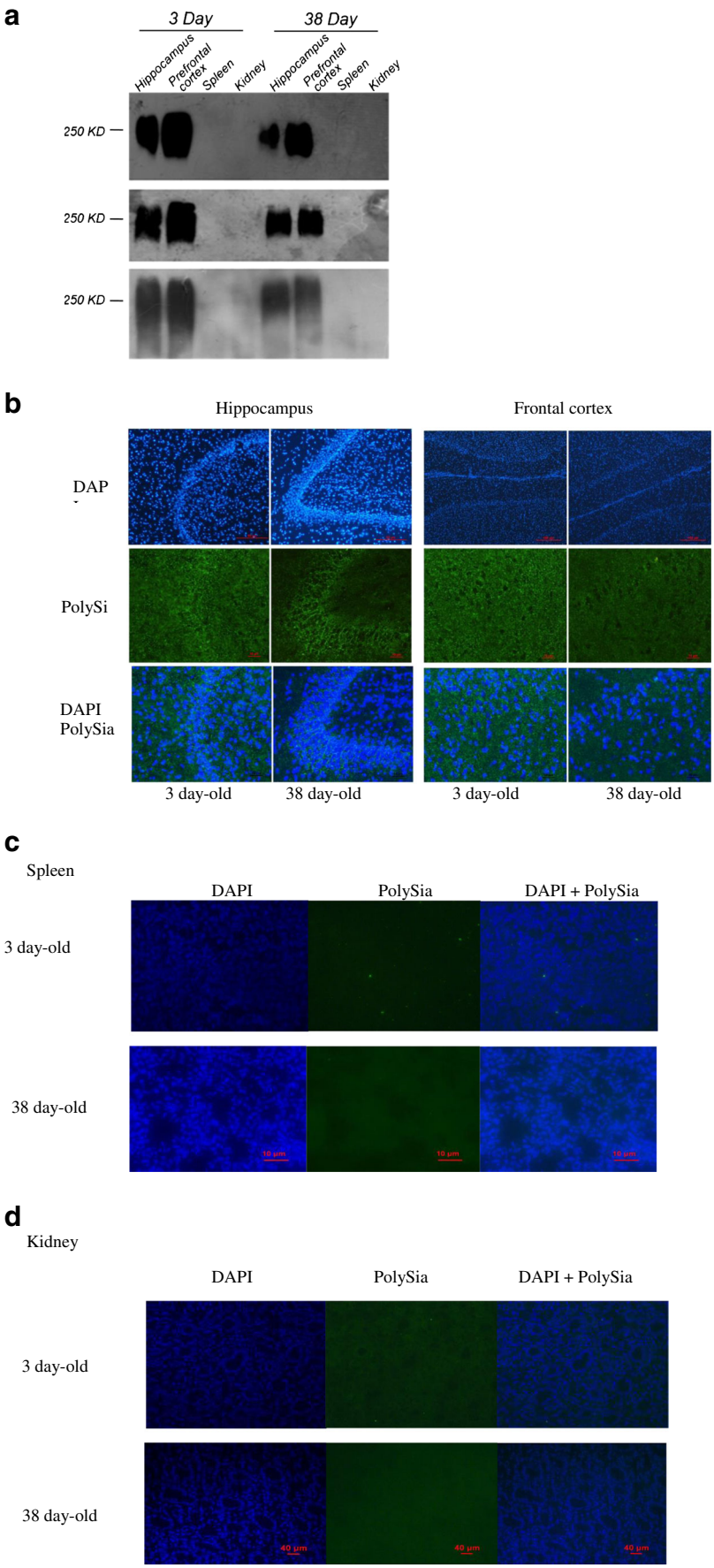

Fig. 5 Western blot and immunohistochemistry analyses of the abundance levels of polySia expressed in postnatal piglet tissues at day 3 and 38. **a** Western blot analysis of the level of polySia in the hippocampus (H), prefrontal cortex (PC) and in non-CNS tissues, spleen (S) and kidney (K); **b**. Immunofluorescent staining of polySia-NCAM (*green*) with DAPI (blue) in the hippocampus and prefrontal cortex in 3 and 38 day-old piglets and the merged images of polySia-NCAM and DAPI (Magnification X40). **c** and **d** Images of polySia-NCAM (*green*) with DAPI (*blue*) staining in kidney (**c**) and spleen (**d**) in 3 and 38 day-old piglets (Magnification X20). These abundance levels were determined using antibodies specific for detecting polySia glycans, as described under "Experimental Procedures"

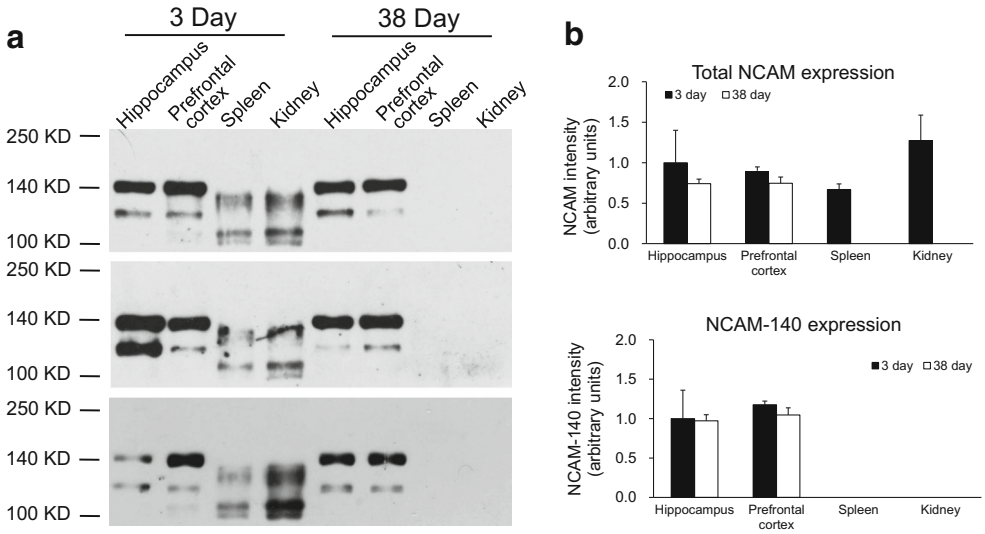

Fig. 6 Western blot analyses of the abundance levels of NCAM proteins in postnatal piglet tissues from three piglets (top, middle and bottom panels) at day 3 and 38. **a** Abundance level of the NCAM protein; **b** Relative comparative level of NCAM-140 expression in the hippocampus, prefrontal cortex, spleen and kidney during postnatal development in 3 and 38 day old piglets. NCAM protein was analyzed with an antibody specific for recognizing the NCAM protein moiety after pretreatment with neuraminidase, as described under "Experimental Procedures"

as model systems. To our knowledge, these findings on the expression of neuropilin-2 during postnatal development in the piglet have not been previously described.

Discussion

The importance of this study is three-fold. First, it describes the cloning of the cDNA for ST8Sia II in the neonatal piglet that has not been previously reported. Second, it reports the quantitative analysis of developmental changes in mRNA expression levels for ST8Sia II and IV, NCAM, neuropilin-2 and the abundance of the cognate α2,8-polySia glycans in neural and non-neural tissues during postnatal development in 3 and 38-day old piglets. Third, the most significant findings is our result showing no correlation between the abundance of message for ST8Sia II and IV and expression level of their cognate posttranslational products, α2-8-polySia. Expression of α2-8-polySia did correlate, however, with expression levels of NCAM and neuropilin-2 in both CNS and non-CNS tissues. NCAM140 and NCAM120 proteins were expressed in the hippocampus and prefrontal cortex at postnatal day 3 and 38. However, in spleen and kidney, immunoreactive NCAM proteins with lower molecular masses (~130 kDa and 120–110 kDa) were expressed at postnatal day 3 but not at day 38 (Fig. 6). These results suggest a down-regulation in NCAM expression in these non-CNS tissues such that no NCAM protein was expressed to function as acceptor substrates for polysialylation, catalyzed by ST8Sia II and IV. These findings underscore again the uncoupling between transcription and posttranslation glycosylation in postnatal piglets given that the mRNA levels coding for ST8Sia II and IV were highly

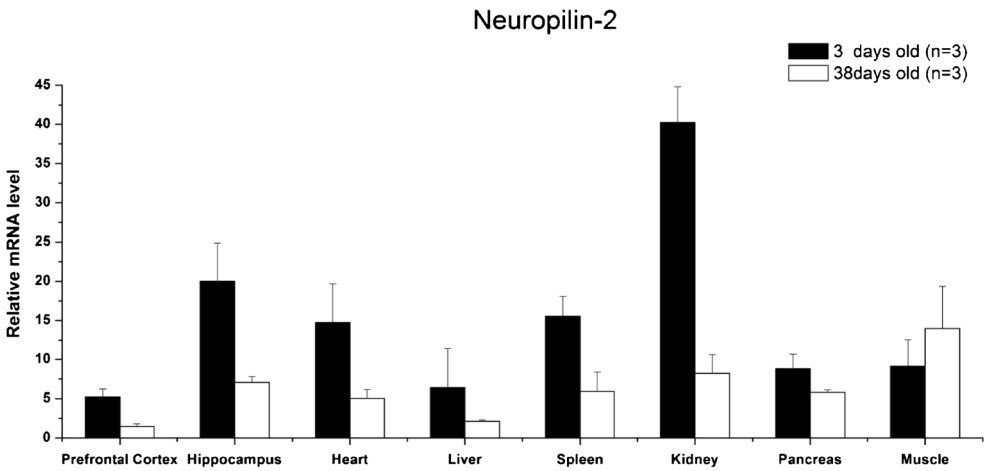

Fig. 7 Relative mRNA expression levels for neuropilin-2 in different neural and non-neural tissues in 3 and 38 day old postnatal piglets. HPRT1 and TBP genes were used as the reference genes. The relative level of mRNA for the individual genes was obtained using a normalization factor that was generated from the double reference genes, using the geNorm software, as described under "Experimental Procedures

expressed in these non-CNS tissues, yet neither of their post-translational cognate α2-8-polySia glycans were detected. In the CNS however, both NCAM and neuropilin-2 proteins were expressed and were sialylated by ST8Sia II and IV. Accordingly, the cellular abundance of ST8Sia II and IV appears to be regulated at the level of translation/posttranslation, and not at the level of transcription.

A further relevant finding is that the NCAM isoforms in the CNS and non-CNS tissues are different in postnatal piglets. In non-CNS tissues, for example, the apparent molecular masses for the NCAM proteins were ~130 kDa and ~110 kDa. The lower molecular mass NCAMs may carry reduced levels of polySia, but perhaps higher levels of α2-8 triSia and α2-8 oligoSia (Zhu, Chen, *et al.* MS in preparation). No DP analyses of these structures have been carried out, however, which is an aim beyond the scope of the present study. An additional new finding is that the mRNA level coding for neuropilin-2 correlates with the expression level of message coding for ST8Sia II and IV in different tissues.

The amino acid sequence deduced from the cDNA encoding for ST8Sia II contained structural features in common with those reported for the polyST in other species, including the short N-terminal cytoplasmic domain, a type II transmembrane domain and a stem region containing a long catalytic domain with the characteristic sialyl motifs (SM) common to the family of sialyltransferases. The novel polybasic polysialyltransferase domain (PSTD) of 32 amino acids immediately upstream of the SMS motif was also present in the piglet ST8Sia II (Fig. 1). Sequence alignment analyses revealed a very close genetic relationship for ST8Sia II between piglet and human (Fig. 2b). This finding is in further support of our supposition that the piglet is a preferred animal model to study the molecular details of how sialic acid/polySia metabolism is regulated, and how these sialylated glycans function in neurodevelopment, cognition and memory in mammalian species. Unlike rodents, the piglet shares similar anatomical structures and physiological features with human infants [2, 48].

Our finding that the mRNA levels for ST8Sia II and IV in piglets declined during postnatal development is similar to that reported in rodents and humans [35]. Interestingly, the level of message for ST8Sia II and IV in the postnatal piglet showed a more rapid decline than message for ST8Sia IV (Fig. 4). It has been reported that ST8Sia II and IV may act coordinately to synthesize polySia during development by differentially regulating their expression at different development stages [26]. ST8Sia II is expressed predominantly during early stages of embryogenesis and diminishes rapidly during development, while mRNA for ST8Sia IV decreases at a slower rate, and is the principale polyST message expressed in adult brain [25, 26, 62, 63]. In contrast, high levels of α2-8-polySia glycans were expressed in both the hippocampus and frontal cortex at day 3, and showed a developmental decline by day 38. This suggests that during postnatal development in the piglet, the cellular abundance of polySia-NCAM in 3 day-old piglets is controlled at the level of translation and not transcription, in accord with the important conclusions of Schwanhausser *et al.* on the quantitative control of mammalian gene expression. [56]. Essentially no polySia was expressed in kidney or spleen at either day 3 or 38 (Fig. 5a, c and d).

Our Western blot analysis to further characterize the relationship between message level for ST8Sia II and IV and their cognate polySia synthetic products also revealed an unexpected finding. We discovered, for example, that the message encoding for polyST's during postnatal development at 3 or 38-days was lower in the hippocampus and frontal cortex, yet was expressed at relatively high levels in the non-CNS tissues (Fig. 4a and b). This is in marked contrast to the high levels of polySia expressed in the hippocampus and frontal cortex at both 3 and 38-days, and its near absence in spleen and kidney during this same period (Fig. 5a, c and d). These findings reveal a clear lack of relationship between mRNA transcript levels and the post-translation levels of the glycans synthesized by STSia II and IV during postnatal development in the piglet.

Surface expression of the anti-adhesive polySia glycans on NCAM disrupts the homophilic binding properties of NCAM, thus attenuating cell adhesive processes during embryogenesis and early development. Elevated levels of polySia-NCAM are abundantly expressed on rapidly developing embryonic neural tissues and decreases as development proceeds. Our present findings showing similar developmentally regulated changes in polySia-NCAM levels in postnatal piglets is in accord with the hypothesis that these changes may likely be a common mechanism among mammalian species. The expression levels of NCAMs were not only higher at postnatal day 3 than day 38 in all tissues, but also the isoform of NCAMs were different between CNS and non-CNS tissues (Fig. 6). Previous studies showed that oligodendrocyte maturation involved polysialylation, implicating a role for polySia in myelination [14]. Therefore, protein carriers of polySia, *e.g.* NCAM and neuropilin-2, have important roles in neurodevelopment, and their expression pattern in cells and tissues are age dependent. Further, we found that expression of neuropilin-2 in different tissues correlated with expression of ST8Sia II and IV, in accord with their function in the polysialylation of neuropilin-2 in postnatal piglets. Recent finding by Rollenhagen, M. *et al*. reported, however, that ST8Sia IV was exclusively responsible for polysialylation of neuropilin-2 on mucin type *O*-linked glycans [15]. Thus, this appears to be another distinction between the mechanism of polysialylation in rodent and postnatal piglets.

In summary, our new findings show that the sub-family of ST8Sia II and IV in neonatal piglets has several features in common with their rodent and human counterparts. These

include similarities in their protein sequence, structural features of their genes and the structure of their cognate biosynthetic products. These characteristics highlight the high conservation of these sialyltransferases among different mammalian species. We also infer from our studies that the neonatal piglet is a preferred animal model to study sialic acid/polySia metabolism in mammals, and to elucidate the molecular mechanisms underlying how sialylated glycoconjugates can enhance neural development, cognition and memory [2, 48]. However, our finding showing a lack of correlation between abundance level of mRNA for ST8Sia II and IV and the abundance level of the post-translation expression of polySia was unexpected. Expression levels of α2-8 polySia did correlate, however, with expression of NCAM and neuropilin-2 protein levels in different tissues. This finding is a further example that the cellular abundance of protein/glycoprotein levels is controlled at the translational/posttranslational levels in postnatal piglets, and not at the level of transcription. This highlights the fundamental differences in the molecular mechanisms controlling polysialylation between rodents and postnatal piglets.*

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Conflict of interest The authors declare that they have no conflict of interests.

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