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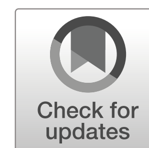

Molecular Mechanisms Underlying How Sialyllactose Intervention Promotes Intestinal Maturity by Upregulating GDNF Through a CREB-Dependent Pathway in Neonatal Piglets

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Abstract

Sialylated milk oligosaccharides (SMOs) have a multifunctional health benefit, yet the molecular details underlying their potential role in modulating intestinal maturation remains unknown. To test the hypothesis that sialyllactose (SL) may mediate intestinal maturation and function through controlling neuronal function, studies were carried out where the diet of postnatal piglets was supplemented with a mixture of 3′- and 6′-sialyllactose from postnatal day 3 to 38. Gene transcription pathways regulating enteric nervous system function, polysialic acid (polySia) synthesis, and cell proliferation were quantified. Our new findings show that SL intervention: (1) upregulated the level of gene and protein expression of the glial-derived neurotrophic factor (GDNF) in the ileum; (2) upregulated phosphorylation of the cAMP responsive element-binding protein (CREB), the downstream target of GDNF signaling pathway; (3) promoted cell proliferation based on an increase in the number and density of Ki-67 positive cells in the crypts; (4) increased the crypt width in the ileum by 10%, while gene markers for the functional cells were not affected; (5) upregulated mRNA expression level of ST8Sia IV, a key polysialyltransferase responsible for synthesis of polySia-NCAM; (6) reduced the incidence and severity of diarrhea. These results show that SL promotes intestinal maturation in neonatal piglets by upregulating GDNF, synthesis of polySia and CREB-interactive pathway.

Keywords GDNF signaling pathway · CREB · Polysialic acid · ST8Sia IV · Enteric nervous system (ENS)

Introduction

Maturation of the intestine during infancy relies entirely on adequate nutritional support. In humans and mammals, the gastrointestinal tract is functionally immature and immune incompetent at birth but undergoes rapid growth and maturation after birth [1]. This developmental process is induced by providing nutrients which, in turn, promotes structural and functional development of the intestine, including digestive and absorptive capacity and intestinal barrier function [2].

Multiple studies have demonstrated the beneficial effects of breast-feeding in preventing morbidity and mortality from necrotizing enterocolitis and diarrhea in both human infants and other mammals. This impacts long-term health and disease, of which inflammatory bowel disease is a serious consequence [3]. The benefits of human milk on intestinal development are related to its nutritional composition and to the biological activities of specific biochemical components. One such class of biochemical or nutritional compounds is the human milk oligosaccharides (HMOs), which are the third largest component in human milk after lactose and lipids, at levels of 20–25 g/L in colostrum and 5–20 g/L in mature milk [4]. Approximately 10~30% of the HMOs are sialylated milk oligosaccharides (SMOs), which is a ~100-fold higher concentration than present in bovine milk or infant formulas [5, 6]. The most abundant human SMOS is sialyllactose (SL), which is found predominantly in two forms, 3′-SL and 6′-

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SL, with the number denoting the position of the Sia monosaccharide linkage to lactose [6]. The concentrations of 6′-SL are the major SL in human breast milk while, in contrast, 3′-SL is the predominant SL in infant formula. The level of 6′-SL in human milk declines during lactation from 500 mg/L at 1 month to 250 mg/L at 2 months, while the concentration of 3′-SL remains relatively constant at ca. 220 mg/L throughout lactation [7]. However, the biological functions of 3′-SL and 6′-SL are similar in supporting resistance to pathogens [8], favoring the growth of beneficial intestinal bacteria [9] and promoting neural function and cognitive development [5]. Molecular mechanism studies underlying the protective effect of SL on intestinal infections, bacterial flora, and maturation have been focused primarily on in vitro studies, including the role of the interaction of the SL motif present in the GM3 ganglioside, its inhibition of the in vitro phosphorylation of the epidermal growth factor receptor [10], and the effect of SL on the growth-related characteristics of intestinal cells [11].

The sialic acids (Sia) are a class of 9-carbon acidic amino sugars that are a marker of SMOs. High concentrations of Sia are found in HMOs, brain gangliosides, sialylated glycoproteins, and the polysialic acid (polySia) glycans, which posttranslationally modifies the cell membrane-associated neural cell adhesion molecules (NCAMs) [12] to mediate a number of critically important cell-to-cell interactions in the brain and immune system, as reviewed in references [13–16].

CMP-Sia (CMP-Neu5Ac) is the activated Sia precursor required for catalyzing synthesis of all sialylated glycans, including the polySia glycan, which posttranslationally polysialylates the cell membrane-associated NCAMs, synaptic cell adhesion molecules (SynCAM), neuropilie2 (NRP2), the α-subunit of the sodium channel [17], CCR7 [18], and the E-selectin ligand [19]. Recently, we reported that dietary intervention with the Sia-rich glycoprotein, lactoferrin (Lf), upregulated intestinal gene expression of the brain-derived neurotrophic factors, BDNF, and alkaline phosphatase activity to alleviate early weaning diarrhea in postnatal piglets [20]. There is, however, a dearth of information on understanding the molecular and cellular mechanisms underlying how sialylated HMOs modulate the enteric nervous system (ENS), intestinal maturation, structure, and function in vivo. The objective of the present study was to determine the molecular and cellular mechanisms underlying how SL modulates the intestinal ENS to promote intestinal maturation in neonatal piglets, a preferred animal model for translational studies relevant for the human infant [21].

Materials and Methods

Neonatal Piglets

Male 3-day-old domestic piglets (*Sus scrofa* landrace × large white F1) were purchased from a commercial pig farm in Xiamen City, China. Based on body weight and litter information, the piglets were randomly divided into the control ($n = 17$) and SL treatment groups ($n = 17$). All animals were housed in pairs in a home pen in a temperature-controlled room (2 °C) with a relative humidity of 50–60% on a 12-h light (08:00–20:00) and dark (20:00~08:00) cycle, as previously described [20]. The home pens contained a "nest," consisting of a rubber tire covered with a clean towel, a heat lamp over the nest, and an identical wooden toy hanging in the home pen. All participating personnel were double blinded as to the diet each piglet received during the study. The experimental protocol was carried out in accordance with guidelines established by the National Natural Science Foundation of China and approved by the Animal Ethics Committee of Xiamen University.

Animal Feeding

3′-SL and 6′-SL (Gene Chem Inc. South Korea) at a ratio of 5:1 were blended into the piglets' milk replacer (Feed & Grow International Co. Ltd. China) at a concentration of 1.71 g/L for the SL intervention group. The group without any supplementation of SL was considered the control group. Quantitative analyses of the total Sia concentration in the final experimental diets were 875 mg/L in the SL treatment group (exogenously added), and 140 mg/L in the control group, which was derived from the piglets' milk replacement (endogenous level). These SL concentrations represent an approximate Sia intake of 240 and 40 mg/kg body weight/day, respectively.

To maintain normal growth rates, the piglets received 285 mL/kg body weight/day from postnatal day 3 to 15, and 230 mL/kg body weight/day for the remaining 23 days. The duration of SL intervention was from 3 to 38 days of age. Thirty-eight-day-old postnatal pigs are approximately equivalent to 10 months old of human infants [21]. Feeding times were at 08:00, 13:00, 18:00, and 22:30 h, with an extra 50 mL milk/pig supplied at the last feeding. Body weights were measured each morning before feeding using a digital scale (PRIS-Scale model: XK 3116, Chengdu Pris Electronic Co. Ltd., China). The milk intake, health status, stool consistency, and any medications were recorded daily. Diarrhea is defined as the passage of three or more loose or liquid stools per day, or more frequent passage than is normal for individual piglets.

Sample Collection

On a postnatal day 38, the piglets were euthanized with sodium pentobarbital (100 mg/kg body). Samples were collected as previously described [20]. In this procedure, the entire small intestine, from pyloric to the ileocecal valve, was immediately removed and the total length measured. Three segments of the small intestine, based on their anatomical location, were collected. Each segment was gently flushed with

diethylpyrocarbonate (DEPC): water (1:1000 *v/v*) at 2–4° C and ~ 50 mg of each tissue section was stored in RNA Later (Invitrogen, USA) for the subsequent quantitative-PCR (q-PCR) analyses. Samples for histological analyses were fixed in a solution of 4% paraformaldehyde. The remaining intestinal samples, and the contents of the colon, were collected and frozen at − 80 °C for subsequent protein and microbiota analyses.

q-PCR Analyses of the Target Gene mRNA Levels in the Ileum

The q-PCR procedure and data analyses were carried out as previously described [20]. In this method, the total RNA was extracted from ileum tissue (10–20 mg) using the Agencourt RNAdvance Tissue Kit (Beckman, USA) and then converted into cDNA following the instruction of RevertAid First strand cDNA synthesis Kit (Fermentas, Canada). q-PCR analyses were carried out using the FastStart Universal SYBR Green Master Mix (Roche, USA) and 7500 real-time PCR (Applied Biosystems). cDNA, corresponding to 50 ng total RNA, served as a template in a 20-μL reaction mixture containing primers and SYBR Green Master mix. Individual samples were measured in triplicate. q-PCR reaction conditions and primer sequences are described in Supplementary Table S1. The relative expression value for each target gene was calculated as a gene quantity divided by a normalization factor (NF), which was derived from GeNorm software (http://medgen.ugent.be/~jvdesomp/genorm/), using GAPDH and TBP as the reference genes. Each primer pair was validated against the reference gene primers with a serial dilution of a reference cDNA sample from the tissues to be analyzed.

Protein Expression Levels in Ileum Analyzed by ELISA and Western Blotting

ELISA Procedures

Approximately 100 mg of full-thickness ileum tissue sections were homogenized in ice-cold HENT buffer (1:10) containing a cocktail of phosphatase inhibitor (PhosStop, Roche) and 1 mM PMSF (phenylmethanesulfonyl fluoride) (Tocris Bioscience, UK) using the Micro Tissue Grinders (T10, IKA, Germany) on ice. Immediately, after homogenization, the homogenates were centrifuged (K30, Sigma, Germany) at 20,000×*g* for 20 mins at 4 °C. Protein expression levels of glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NTF3), and GDNF family receptor alpha 1 (GFRα1) in the supernatant fractions were measured by specific ELISA kits (Elabscience, China), in accordance with manufacturer's instructions. The protein concentration in each sample was determined using the BCA protein assay (Pierce BCA Protein Assay Kit, USA). All analyses of the ELISA and protein assays were carried out in triplicate. The values determined for GDNF, BDNF, NTF3, and GFRα1 were normalized to that of the protein concentration.

Western Blotting Procedure

Western blotting for the quantitative analyses of the expression level of target proteins was carried out as described previously [21]. Equal amounts of proteins (60 μg) were resolved by SDS/PAGE electrophoresis using 10% polyacrylamide resolving gel (CREB, phospho-CREB ERK1/2, and phospho-ERK1/2) or 7% for polySia, while 8% polyacrylamide gels were used for resolving Akt, phospho-Akt, ribosomal S6 kinases (RSKs), p90, and phospho-RSK p90. The separated proteins were transferred electrophoretically onto a PVDF membrane at 90 V for 1.5 h. The membrane was first blocked by incubation with TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (*v/v*)) containing 5% skimmed milk for 2 h at room temperature with gentle shaking. The membranes were then incubated overnight at 4 °C with primary antibodies to mouse anti-polySia (1:1000, mAb 735), rabbit anti-CREB and phospho-CREB (1:500, cell signaling technology), Akt and phospho-Akt (1:1000, cell signaling technology), RSK1 p90 (1:500, Abcam), phospho-RSK p90 (1:500, cell signaling technology), and ERK1/2 and phospho-ERK1/2 (1:2000, cell signaling technology), respectively (Supplementary Table S2). After extensive washing in TBST, the membranes were incubated with secondary antibody (horseradish-peroxidase-conjugated anti-rabbit 1:10,000, Sigma) at room temperature for 2 h. Proteins on the blots were detected using enhanced chemiluminescence (CW0049A, CWBIO, China; RPN2235, GE Healthcare, USA), after exposure to X-ray films (XBT-1, Kodak, USA). A mouse primary antibody to β-actin was used as a control for the amount of protein loaded on each gel and detected using an anti-β-actin mAb (A1978, 1:1000, Sigma, USA). Quantitation of the protein bands was carried out by scanning the films using the *Image Analyses Software* (Quantity One, Bio-Rad, USA). The density of protein bands on all films was determined under non-saturating conditions.

Intestinal Histological Analyses

Paraformaldehyde-fixed samples of jejunum and ileum were dehydrated overnight in 30% sucrose, embedded in OCT (SAKURA, Japan), sectioned in 5-μm thicknesses in a cryostat microtome (LEICA CM 1950, Germany) before being mounted on slides. For measurement of villus-crypt structures, the slides were stained with hematoxylin and eosin. The villus area, from the tip of the villi to the villus/crypt junction, and the crypt area were measured at × 100 magnification using an Olympus microscope (Olympus IX 5, Japan)

and Imagepro Plus software, in 12–15 vertically well-orientated villus and crypts [22]. All sample analyses were carried out blinded, such that the nature of the results remained unknown to the investigators.

Intestinal Immunofluorescence Assay

Immunofluorescent analyses of the jejunum and ileum were carried out on 5-μm thick slices on glass slides. After the slides were first rinsed with water, they were incubated with 1 mg/mL sodium borohydride in phosphate-buffered saline containing Tween 20 (PBST, 0.01 mol/L, pH 7.4) 3 times for 10 mins at room temperature. After washing with PBST two times for 5 mins each, the slides were incubated with a primary rabbit, anti-Ki67 antibody (1:200, ab15580, Abcam, USA) in an antibody dilute buffer (AR1017, Bo Shide, China) overnight at 4 °C. The slides were then washed 3 times for 10 mins each with PBST at room temperature and then incubated with secondary antibody (1:200, A11012, AlexaFluor 594, Life technology, USA). After 2-h incubation at room temperature, the slides were washed 3 times with PBST for 15 min each at room temperature. Control sections in which either primary antibodies or secondary antibodies were omitted showed no labeling of cells. All sections were counterstained with DAPI (VECTA SHIELD®). The images obtained were analyzed using a fluorescence microscope (Olympus IX 5, Japan). Cells in the ileum positive for Ki 67 were counted using Image-Pro Plus software. Total quantifications were carried out on six-independent intestine samples from each group of piglets, and at least 30 crypts per sample were analyzed.

Analyses of Stool Consistency

Visual assessment of stool consistency was assessed each morning using a modified method as described previously [20]. The detail methods of stool consistency assessment are described in Supplementary Material.

Statistical Analyses

Comparison of the expression level of target genes and their cognate proteins, intestinal histology, and K67 staining was carried out using Univariate ANOVA for normal distribution or a Tweedie generalized linear model for non-normal distribution. Differences in the incidence and severity of diarrhea were compared using a Binomial or Tweedie generalized linear model with a probiotic (Medilac-Vita, from a local pharmacy) as a covariate. Differences in the onset of diarrhea were compared using the Kaplan–Meier survival analysis. Normality was assessed using the Kolmogorov–Smirnov test. Data are expressed as mean ± SEM, and a significance level of 0.05 was used. All statistical analyses were confirmed using the SPSS for Windows 19 Inc. Chicago, IL.

Results

Sialyllactose Upregulates the GDNF Neurotrophic Signaling Pathway in the Ileum

GDNF is a member of a family of critically important neurotrophic growth factors and a key neurotrophic factor expressed in the postnatal enteric nervous system (ENS) [23]. GDNF is expressed in the embryonic intestine and activates the transmembrane receptor tyrosine kinase, Ret, and a co-receptor GDNF family receptor α1 (GFRα1), which promotes the development of ENS during embryonic development [24]. Our present findings show that the mRNA level for GDNF was upregulated 86% in the SL intervention group, compared with the control group ($P = 0.03$, Fig. 1a). The NTF3 gene expression level, however, was not significantly different between the SL intervention group and the control group of piglets ($P > 0.05$). Accordingly, the protein expression level of GDNF in the SL intervention group was significantly increased in the ileum compared with the control group of piglets ($P = 0.02$, Fig. 1b). The protein expression levels of NT3, BDNF, and GFRα1 were not altered by SL intervention (Fig. 1b).

GDNF signaling stimulates the phosphorylation of CREB-1, ATF-1, and CREM-1, and c-fos transcription through a multicomponent receptor complex [25]. To determine the effects of upregulated GDNF expression on the intracellular activation of its downstream proteins, we examined the functional consequences of the SL-induced message levels for CREB phosphorylation (pCREB) and p90 RSK using Western blotting. Our results show that pCREB at Ser-133 was significantly upregulated in the SL intervention group compared with the control group ($P = 0.007$, Fig. 1c), indicating activation of the GDNF downstream signaling pathway. Recent in vivo studies have demonstrated that CREB and the CREB-binding protein (CBP) are required for intestinal stem cell (ISC) survival while overexpressing CREB promotes cell proliferation [26]. Similar to what was observed with CREB, the phosphorylation of RSKs, the major CREB kinases, showed a 14% increase in the SL-treated piglets compared with the control (Fig. 1c).

We also determined the protein expression level of the GDNF family receptor alpha 1 (GFR α1) and found no significant difference between the SL intervention and control groups of piglets (Fig. 1b). We further examined the activation levels of the extracellular signal-regulated kinases (ERK), the ERK downstream proteins, p90 RSK, and Akt (serine/threonine-specific protein kinase, also known as protein kinase B), which are involved in downstream effectors of GDNF/RET.

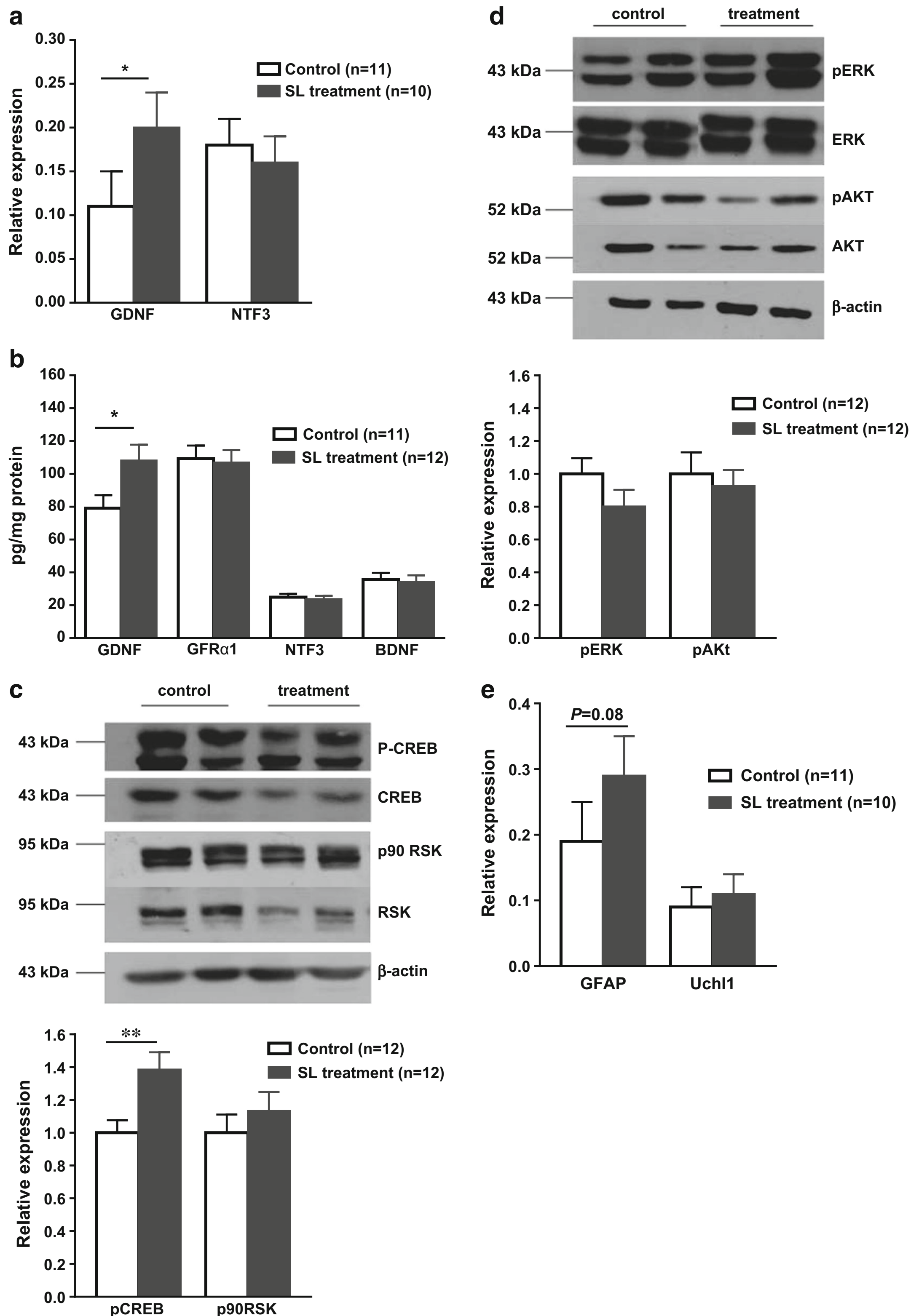

Fig. 1 Sialyllactose intervention upregulates neurotrophin factors and CREB in the ileum of neonatal piglets. **a** mRNA levels of GDNF ($P = 0.03$), NTF3 ($P = 0.74$). **b** Protein expression levels of GDNF ($P = 0.02$), GFRα1, NTF3 ($P = 0.18$), and BDNF ($P = 0.80$). **c** Western blot analyses of the abundance levels of phospho-CREB and phospho-p90RSK, the relative comparative level of phospho-CREB ($P = 0.007$), and phospho-p90RSK after normalization to the abundance level of β-actin. **d** Western blot of the abundance level of phospho-ERK and phospho-Akt, the relative comparative level of phospho-ERK, and phospho-Akt after normalization to the abundance level of β-actin. **e** mRNA expression level of GFAP ($P = 0.08$) and UchL 1($P = 0.17$). Differences between groups were analyzed using univariate ANOVA for normal distributed data or the Tweedie generalized linear model, $^{*}P < 0.05$, $^{**}P < 0.01$

However, no significant differences were found in the protein expression levels of pERK p90 RSK and pAkt between the SL intervention and the control group of piglets (Fig. 1d). The molecular events linking cell surface receptors to the activation of ERKs are complex, with many different stimuli that can activate the ERKs. For example, GDNF-stimulated PI3K and Akt activities exert opposing effects on the ERK pathway. RAS/ERK and PI3K signaling pathways are important for activation of CREB in GDNF-treated cells, respectively [27].

To examine if other neurotrophic factors also contributed to the effects of SL intervention on ENS and gut development, we analyzed the gene expression levels of two other major neurotrophic factors, BDNF and NTF3 in the ileum. Our findings showed no statistically significant differences between the SL intervention and control piglets ($P > 0.05$, Fig. 1b). In contrast, the expression level of the enteric neuron marker, UchL1, and the enteric glial cells marker, GFAP, was upregulated 22% and 53%, respectively in the SL intervention group of piglets compared with the control group. The overall difference, however, was only marginally significant ($P = 0.17$ and $P = 0.08$, respectively, Fig. 1e).

Sialyllactose Intervention Upregulates the Polysialyltransferase, ST8Sia IV (PST) but Not ST8Sia II (STX) in the Ileum of 38-Day-Old Piglets

In all mammalian cells, synthesis of polySia is catalyzed by two polysialyltransferases, designated, ST8Sia II (STX) and ST8Sia IV (PST), during fetal and early postnatal brain development. ST8Sia IV is the principal polysialyltransferase expressed in the postnatal and adult brain [28]. Our results show that the level of ST8Sia IV gene expression was upregulated by SL intervention in the ileum ($P = 0.01$, Fig. 2a), while expression of ST8Sia II was only marginally responsive to SL ($P = 0.09$, Fig. 2a). We view this unexpected finding significant because it provides new evidence for directly linking our present study to our earlier neurobiological results by connecting the brain and intestine, likely through the gut–brain axis via the vagus nerve. The oligosialyltransferase, ST8SiaIII, which catalyzes synthesis of the α-2,8-triSia moiety expressed in gangliosides and several neural glycoproteins [29], was slightly downregulated in the ileum with SL intervention compared with the control (Fig. 2a).

Gene expression levels of the major protein carriers of polySia, viz. NCAM, synaptic cell adhesion molecule (SynCam1), and NRP 2, were upregulated by 23%, 10%, and 38% respectively, in the SL intervention group of piglets compared with the control group (Fig. 2b). In postnatal piglets, polySia could be detected within the ileum and was slightly increased (~ 12%) in piglets whose diets were supplemented with SL, compared with the control piglets (Fig. 2c).

Effect of Sialyllactose Intervention on the Level of Gene Expression Markers in Enteric Cells of the Ilium

The effect of SL intervention on the level of gene expression in four functional cell markers, chromogranin A (ChGA), alkaline phosphatase (IAP), mucin 2 (Muc2), and lysozyme (LYZ), in the ileum, was quantitatively determined using q-PCR (Fig. 3a, b). The mRNA levels for the cell markers of enterocytes (IAP), goblets cells (Muc2), and Paneth cells (LYZ) were upregulated by 10%, 26%, and 110%, respectively following SL intervention compared with the control group (Fig. 3a). No significant difference was found in the gene marker for neuroendocrine cells (ChGA) (Fig. 3a).

We also determined the effect of SL intervention on intestinal cell survival by examining changes in the expression level of cell regulatory molecules, including caudal-type homeobox 2 (CDX2) and epidermal growth factor receptor (EGFR). As shown by our q-PCR results, the mRNA levels of CDX2, a critical regulator of intestinal cell proliferation and intestinal epithelial maturation [30], were upregulated ca. 25% by SL intervention. EGFR, a marker of cell proliferation, increased by ca. 31% in the SL intervention group of piglets ($P = 0.09$, Fig. 3b).

Effect of Sialyllactose Intervention on the Protein Expression Level of Ki67: a Marker for Cell Proliferation

In the intestinal crypts of piglets, epithelial stem cells and multipotent progenitor cells were proliferating and showed nuclear staining for Ki67, a marker for cell proliferation [31] (Fig. 4a). Quantitative analyses showed that the number of $Ki67^{+}$ proliferating cells per crypt was significantly higher in the SL intervention group ($P = 0.04$, Fig. 4b), and the number of $Ki67^{+}$ proliferating cells per crypt depth was also increased by dietary SL intervention ($P = 0.03$, Fig. 4c). Thus, these results demonstrate that dietary SL intervention enhances cell proliferation in the intestinal crypts of neonatal piglets.

Effect of Sialyllactose Intervention on Intestinal Histomorphology

H&E stained sections of the jejunum and ileum of neonatal piglets showed that piglets whose diet was supplemented with SL had a 16% larger crypt area and a 7% larger crypt depth in the ileum, compared with the control group (Fig. 5a, b). In addition, after SL intervention, the crypt width in the ileum increase by ca. 10% compared with the control group ($P = 0.08$, Fig. 5c). However, SL intervention had no effect on the area of the intestinal villus, villus height, or the villus height and crypt depth ratio (Fig. 5d–f).

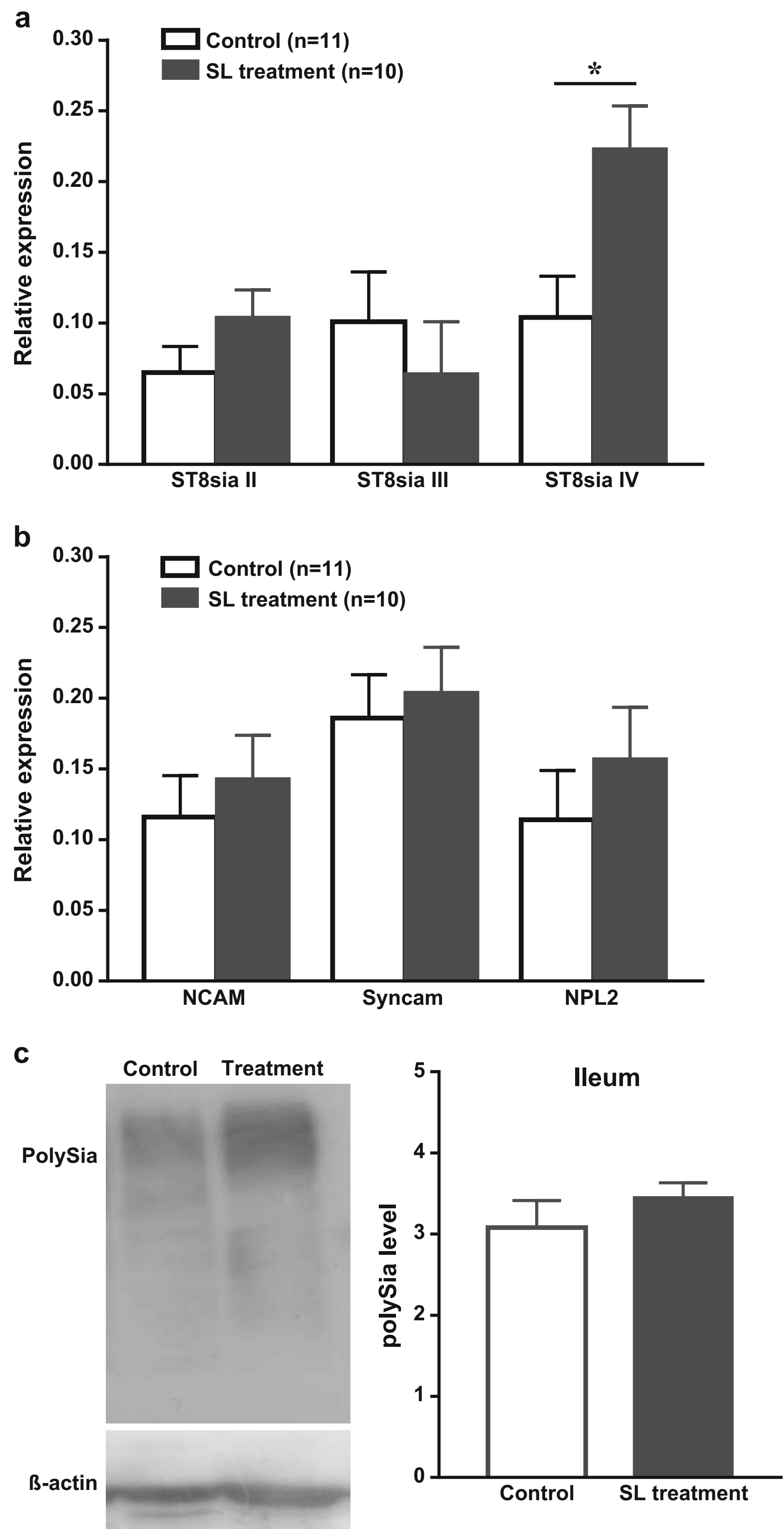

Fig. 2 Comparison of the mRNA expression levels of the polysialyltransferases, ST8Sia II and ST8Sia IV, the oligosialyltransferase, ST8Sia III, and the polySia-carrier proteins in the ileum between the sialyllactose intervention and control groups of 38-day-old neonatal piglets. **a** mRNA level of ST8Sia II ($P = 0.09$), ST8Sia III ($P = 0.93$), ST8Sia IV ($P = 0.01$). **b** mRNA level of NCAM ($P = 0.15$), SynCAM ($P = 0.25$), NRP2 ($P = 0.15$). **c** Representative Western blot and relative quantification of polySia expression. Differences between the two groups were analyzed using univariate ANOVA, $^*P < 0.05$

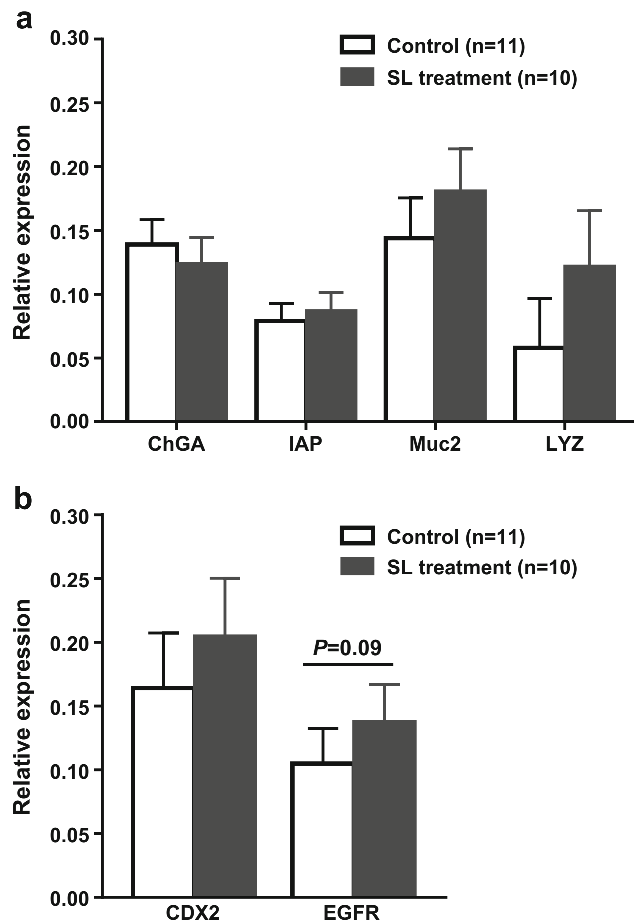

Fig. 3 Effects of sialyllactose intervention on four functional cell markers between SL intervention piglets and the control group of 38-day-old piglets. **a** mRNA level of the four functional intestinal cell markers: chGA, IAP, Muc2, and LYZ. **b** Intestinal maturation markers: CDX2 and EGFR. Differences between the two groups were analyzed using univariate ANOVA

Sialyllactose Intervention Protects Piglets from Weaning Diarrhea

Diarrhea is the manifestation of a disturbed intestinal environment, characterized by nutrient malabsorption and dehydration, which can impose long-term and irreversible consequences on the infants' well-being. In the present study, we found that SL intervention significantly reduced the incidence of diarrhea in piglets during the first 38 days following birth (Fig. 6a, $P = 0.046$, binomial generalized linear model, with probiotic as a covariate). However, the onset of diarrhea between the SL intervention and control group of piglets showed no significant difference ($P = 0.17$, Fig. 6b). We also analyzed the severity of diarrhea using a fecal consistency score based on a scale from 0 to 3. Our findings showed a ~24% reduction in the severity of diarrhea in the SL intervention group of piglets, compared with the control group ($P = 0.03$, Fig. 6c).

Discussion

The presence of SMOS in human milk has been shown to be a significant health benefit for the neonate. For example, orally administered SMOS promotes growth and metabolic changes indicative of improved nutrient utilization [32]. Although the role of milk SL on intestinal development has been extensively studied, the molecular and cellular processes underlying its function in protecting the neonate are poorly understood. To better understand these molecular processes, we administrated 3′-SL and 6′-SL to neonatal piglets at a critical stage in their intestinal maturation from 3 to 38 days of age. For several reasons, the neonatal piglet is the preferred and well-accepted preclinical animal model for translational studies applicable to the human infant, particularly for studies relative to intestinal growth and function and cognitive neurodevelopment [33]. Our present findings have shown that SL intervention upregulated the GDNF neurotrophic factor-signaling pathway for both transcription and posttranslation in the ENS, and the intrinsic innervation of the gastrointestinal tract. GDNF is a key neurotrophic factor in the control of neuron survival and differentiation of postnatal enteric neurons [34] and is involved in the self-renewal of epithelial cells [35]. Previous studies have suggested that during intestinal development, GDNF is expressed in the intestine and its signaling to RET- and GFRα1, genes encoding a member of the GDNF receptor family of proteins, that directs the development of the ENS during embryonic development [36]. More recently, it has been reported that the pleiotropic function of GDNF is, in part, governed by modulating the level of the intracellular activation of the GDNF downstream pathways [23].

We have further characterized the GDNF signaling pathway to determine the status of the downstream proteins in the extracellular signal-regulated kinases, ERK, and the CREB, and the phosphorylated CREB, pCREB, using qPCR and Western blot analyses. CREB, a transcription factor downstream of GDNF signaling, is recognized as a critical regulator of gene expression in the central nervous system (CNS) and the ENS [37], because it contributes to neuronal plasticity, long-term potentiation, memory, fear conditioning, circadian rhythm entrainment, and neuron survival [38]. Our findings thus show that the level of pCREB at Ser-133 was upregulated in neonatal piglets by SL intervention compared with the control group of piglets. These results suggest that SL intervention activates the GDNF downstream signaling pathway, and results in the activation of the transcription factor, CREB, to optimize intestinal development and maturation in neonatal piglets. However, we did not find that SL intervention activated the GDNF receptor, GFRα1, or the extracellular signals, ERK or AKT. There was no significant effect of SL intervention on ERK may be because ERK is involved in many extracellular signaling pathways, including a range of growth factors, G protein-coupled receptors (GPCRs), and iron status

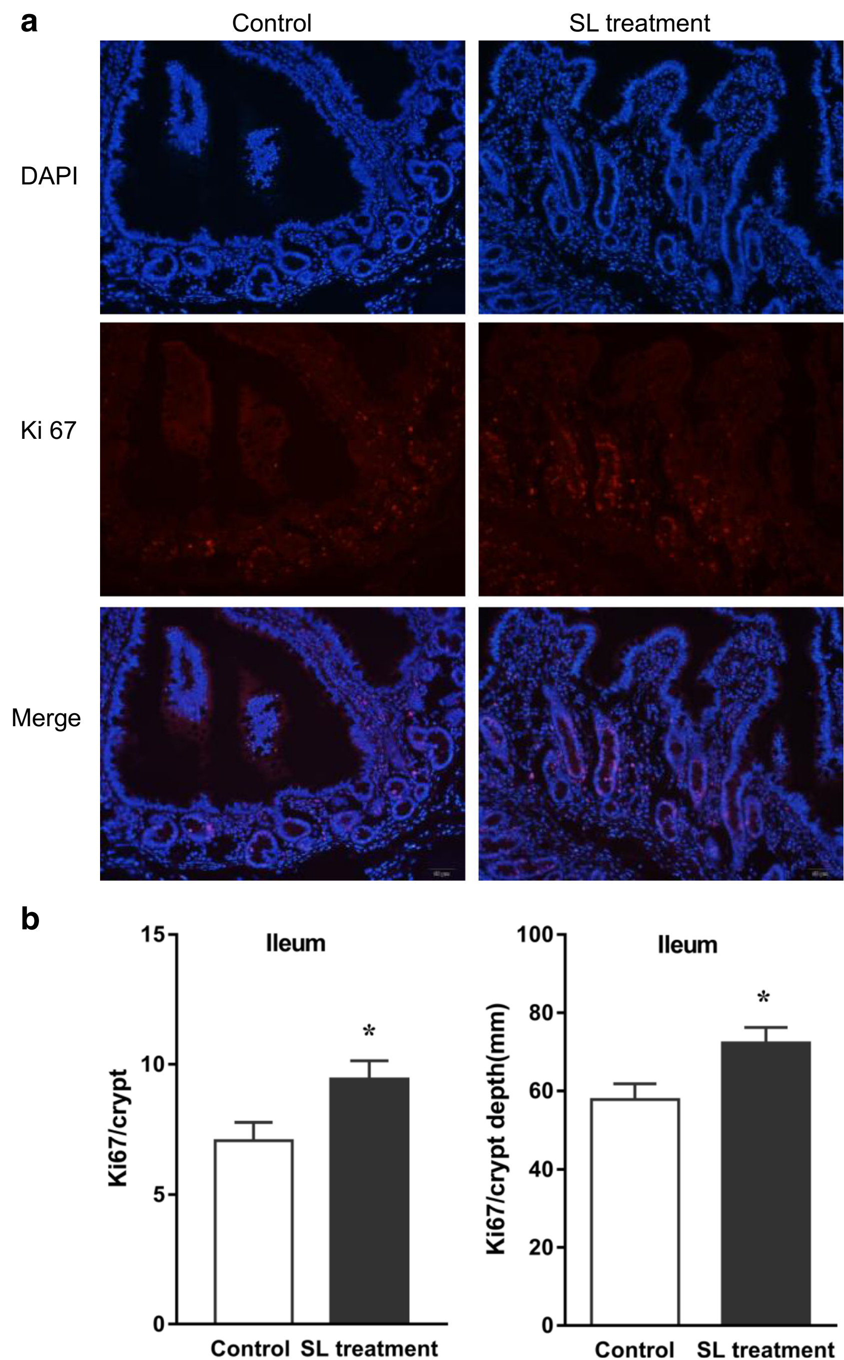

Fig. 4 Sialyllactose intervention increased the level of cell proliferation marker of Ki67 in the ileum of 38-day-old piglets. **a** Immunofluorescent staining of Ki67 showed increased proliferation activity in the ileum of SL intervention group of neonatal piglets in comparison to the control group (DAPI blue, Ki67 red). **b** Number of Ki67-positive cells per crypt (P = 0.047). **c** Number of Ki67-positive cells per length of ileum crypt (P = 0.035). Images are representative of 6 piglets per group. Original magnification was (× 100). Results are expressed as mean ± SEM (n = 6 animals per group). Differences between the 2 groups were analyzed using univariate ANOVA, *P < 0.05

[39]. Although GFRα1 is the main GDNF receptor, the family of GDNF receptors also includes GFRα24 and GDNF family receptor alpha-like may contribute to activation of GDNF signaling pathway by SL intervention. Further, GDNF signaling may operate through activation of polySia-NCAM to initiate activation of the proto-oncogene tyrosine-protein kinase, Fyn, leading to the phosphorylation of CREB [40]. PolySia-NCAM mediated mRet-independent GDNF signaling and can also lead to downstream FAK activation [41]. We found that the level of polySia in the ileum was ~ 12% greater in the SL intervention group of neonatal piglets compared with control piglets. Activation of pCREB in the ileum implies that it may be involved in optimizing intestinal development by SL intervention, a new finding that has not, to our knowledge, been previously described.

SL can be hydrolyzed by neuraminidases during paracellular transport, presumably via the vagus nerve, and the dietary Sia can be transported via blood, to the brain and other tissues, where it is utilized in the Golgi-dependent glycosylation pathway for synthesis of CMP-Sia, the activated precursor of Sia required for sialylation of all sialylated glycans, including polySia, which posttranslationally modifies NCAM, SynCAM, and NRP2 [28].

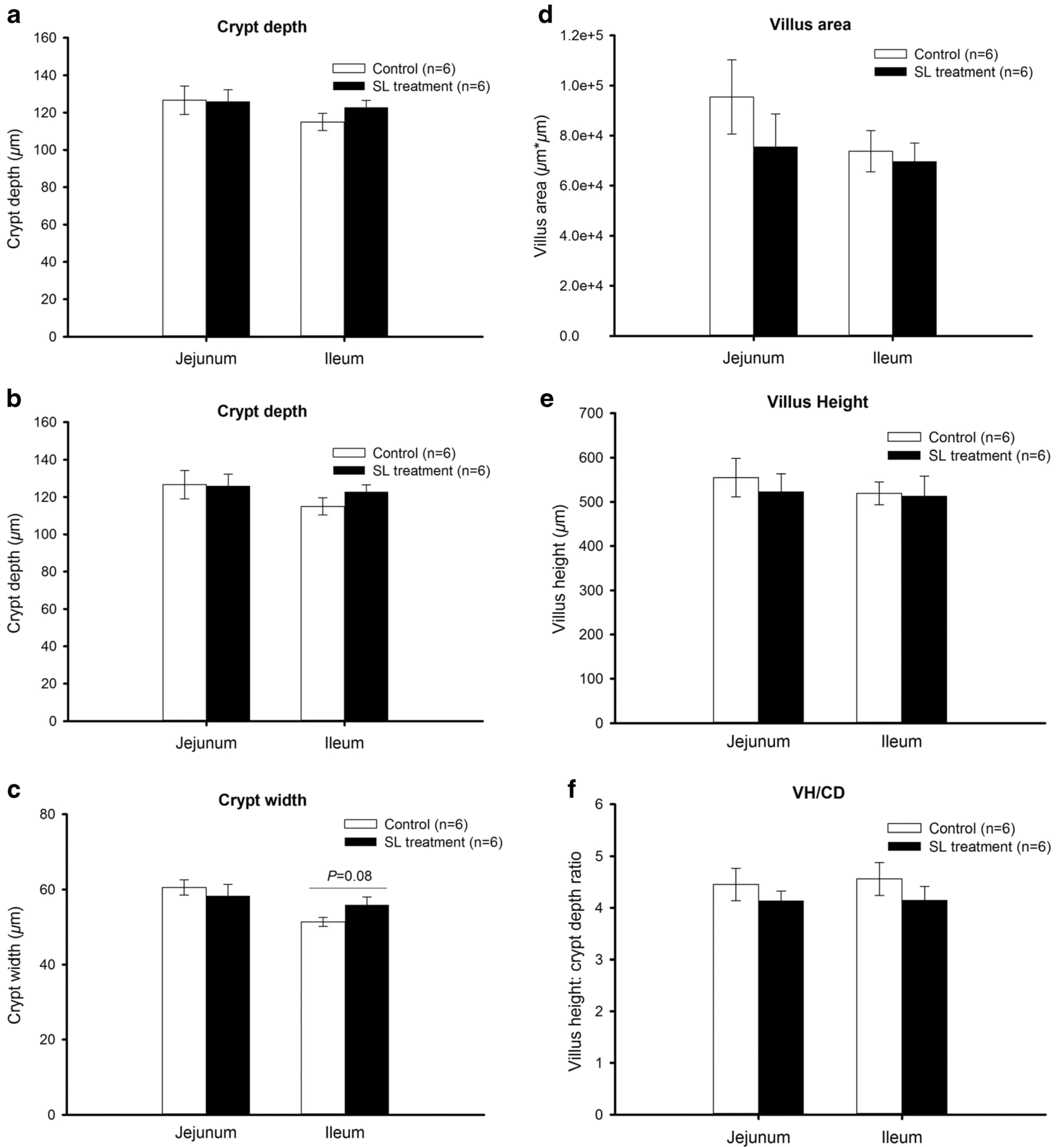

Fig. 5 Effect of sialyllactose intervention on the histomorphology of the jejunum and ileum in the neonatal piglets. **a** Crypt area, **b** crypt depth, **c** crypt width, **d**. villus area, **e** villus height, **f** VH/CD; values are means ± SEM. The villus-crypt structures were measured at × 100 magnification using an Olympus microscope. Differences between the two groups were analyzed using univariate ANOVA

In the brain, polysialylation of NCAM is catalyzed by one of the two polysialyltransferases, either ST8Sia II or ST8Sia IV, as noted above PolySia-NCAM functions in a number of critical roles to modulate cell–cell interactions important for neuronal outgrowth, synaptic connectivity, learning, and memory formation [42]. Our present findings show that the transcription levels of ST8Sia IV in the ileum of neonatal piglets were upregulated > twofold by SL intervention in the intestine of 38-day-old piglets ($P < 0.05$), while ST8Sia II was increased only ~30% compared with the control group of piglets. These findings confirm our previous published studies showing that Sia intervention significantly increased the

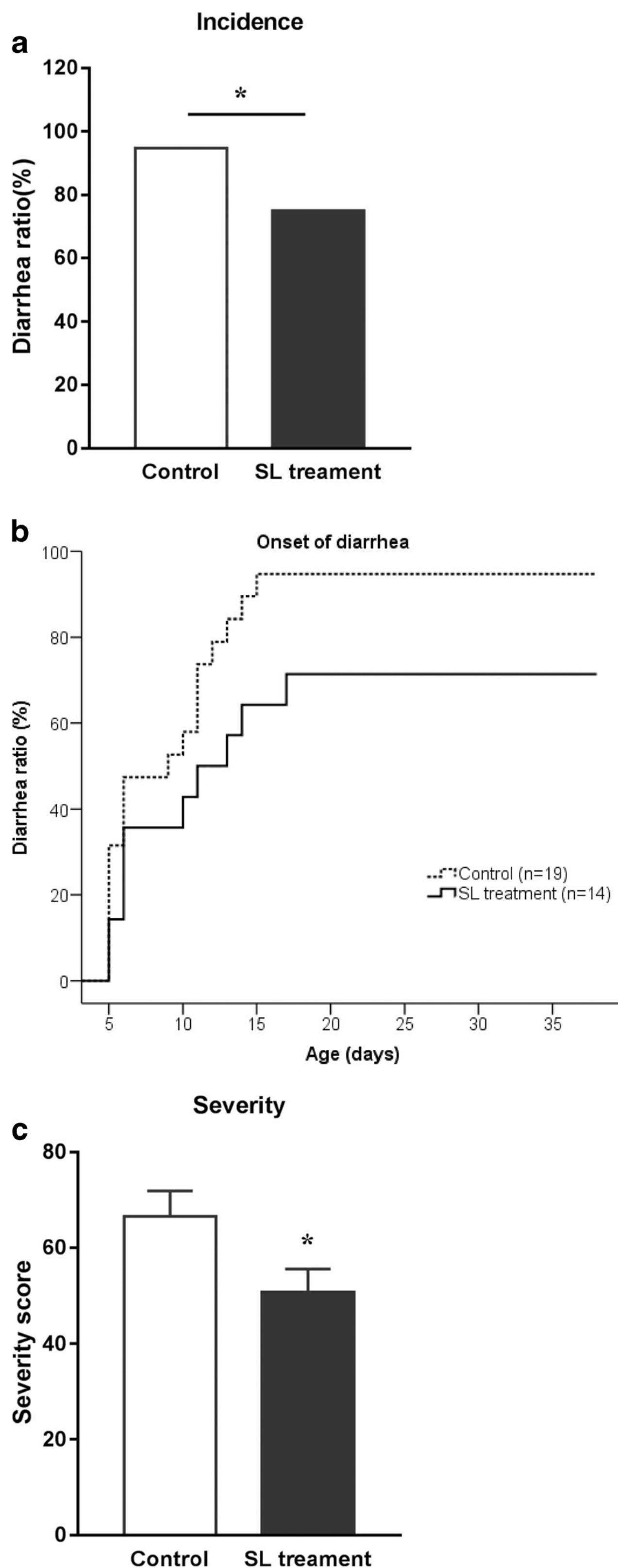

Fig. 6 Sialyllactose intervention prevented weaning diarrhea in neonatal piglets. **a** Reduced incidence of weaning (non-infectious) diarrhea ($P = 0.046$). **b** Onset of weaning diarrhea ($P = 0.03$, Cox regression with medication as covariance). **c** Severity of weaning (non-infectious) diarrhea ($P = 0.03$). The statistical analyses of panels (**a**) and (**c**) were carried out using the generalized linear model as described in the text, $^{*}P < 0.05$

mRNA levels in the hippocampus encoding for ST8Sia IV in developing piglet [43]. Interestingly, the increased transcriptional level of ST8Sia IV and ST8Sia II resulted in a ~12% increase in polySia in the ileum tissue in developing neonatal piglets. As we have described previously, there is a lack of correlation between transcription levels of ST8Sia II and ST8Sia IV and the posttranslational levels of polySia-NCAM in different regions of the piglet's brain during postnatal development [44, 45]. Thus, our present studies show that the cellular levels of mRNA encoding for ST8Sia II and ST8Sia IV, genes encoding for several proteins, and the level of polySia expression are also tissue specific.

ST8Sia III, an oligosialyltransferase principally responsible for the synthesis of the di- and triSia glycans on both neural gangliosides and several neural glycoproteins, was downregulated by ca. 50% following SL intervention. These findings suggest that SL intervention may thus modulate polySia glycan synthesis to promote the migration of enteric precursors during the development of ENS, in contrast to di- and triSia glycans synthesis. Therefore, we conclude from these findings that SL modulates polySia glycan synthesis not only in the brain but also in the intestine. To our knowledge, this unexpected finding has not been previously reported and is significant because it links neural/brain function with and intestinal maturation.

Development and maturation of the ENS persist in postnatal life, and the plasticity of the postnatal ENS is influenced by neurotrophins, including BDNF, NTF3, and GDNF. While GDNF is a critically important neurotrophin for postnatal enteric neuronal survival and plasticity; the enteric glia cells are involved in most intestinal functions, including digestion and absorption, motility, mucosal secretion, and host defense [46]. Previous cell culture studies have reported that SL intervention may also affect intestinal epithelial cell proliferation and induce differentiation in epithelial crypt cells [11]. More recently, Hoischer et al. used HT-29 and Caco-2Bbe cell lines as models of crypt–villus axis and reported that 6′SL caused a dose-dependent inhibition of cell proliferation in undifferentiated HT-29 cultures and well-differentiated Caco-2Bbe cells [47]. Importantly, however, there are no reported in vivo studies designed to study the molecular processes underlying the effect of SL intervention on intestinal cell maturation and digestive functions.

As intestinal proliferation is a primary factor for intestinal growth and development, we analyzed the role of SL intervention on intestinal proliferation in vivo using the Ki67 immunofluorescence assay. Our results show that SL intervention in neonatal piglets increased intestinal crypts proliferation at 38 days after birth by 33%. In addition, the level of mRNA expression for CDX2 and EGFR, critical regulators of intestinal epithelial maturation [30], was upregulated 25% and 32%, respectively after SL intervention, compared with the control group of neonatal

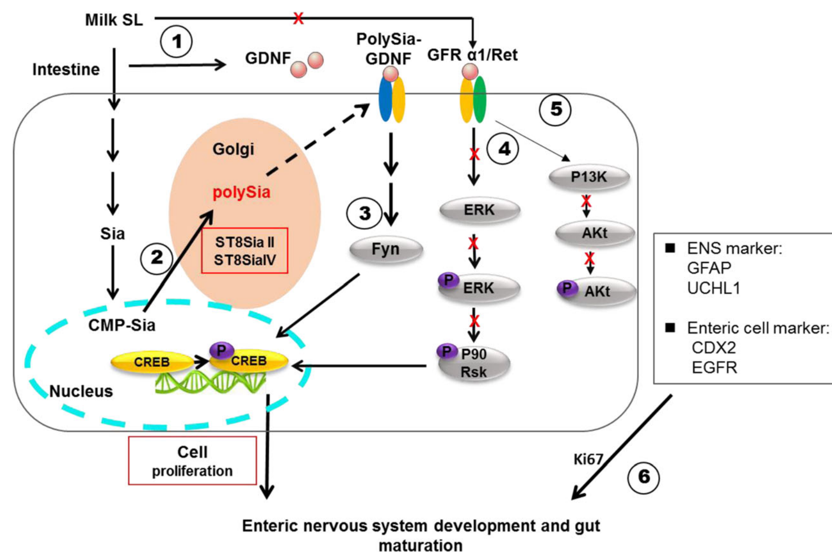

Fig. 7 Proposed pathway and molecular mechanism by which milk sialyllactose intervention may influence intestinal maturation. (1) Exogenous SL upregulated the expression level of mRNA and protein for GDNF in the ileum; (2) dietary SL intervention also provides a major source of Sia to activate gene expression levels of ST8Sia IV for the synthesis of polySia on NCAM in the ileum. (3) PolySia mediates binding to GDNF, which activates Fyn to increase expression levels of the phosphorylation of CREB [41]. (4–5) SL intervention did not directly activate GFRa1, one of GDNF receptor and did not activate the downstream proteins of ERK, pERK, p90 RSK, and Akt/pAkt, Therefore, the upregulated levels of GDNF gene and protein expression results in upregulated phosphorylation of CREB were not mediated through activation of the Ras/ERK1/2 signaling pathway. (6) GDNF plays a pivotal role in regulating postnatal ENS development and epithelial cell fate [46] through upregulating the level of the transcription factor, phospho-CREB and to promote cell proliferation, evidence by an increase in the number and density of Ki-67 positive cells in the crypts. SL, 3′- and 6′-sialyllactose; GDNF, glial cell line-derived neurotrophic factor; UchL1, ubiquitin carboxy-terminal hydrolase L1; ST8Sia IV, polysialyltransferase IV; NCAM, neural cell adhesion molecules; polySia, polysialic acid; polySia-NCAM, polysialylated NCAM; BDNF, brain-derived neurotropic factor, ENS, enteric nervous system

piglets. Collectively, these findings show that SL intervention increased expression of the intestinal crypt proliferation marker, Ki67, in the ileum, promoted crypt and villus integrity, and also increased intestinal epithelial cell proliferation. Accordingly, compared with the previous finding that SL intervention may inhibit intestinal epithelial cell proliferation and induce differentiation in epithelial crypt cells in vitro [11], our new in vivo findings lead us to postulate that additional molecular processes, other than the direct effect of SL on epithelial cells, mediates the effects of SL intervention on intestinal cell proliferation in neonatal piglets. Significantly, this is the first study to investigate the molecular mechanisms of SL intervention on intestinal proliferation in neonatal piglets.

The early weaning transition is commonly associated with increased occurrence of enteric diseases and diarrhea. Although the ability of SL intervention to protect against infection and diarrhea was first attributed to its attachment to pathogenic bacterial species [48], there is increasing evidence that the sialyl moiety of SL may directly modulate the intestinal cell properties [47] and enhance the development and function of the CNS [49]. Further, our study shows that SL intervention can significantly improve gut comfort during weaning by reducing the incidence and severity of early weaning diarrhea in postnatal piglets provides the first in vivo evidence for the protective effect of SL intervention on early weaning diarrhea in piglets. Additionally, intestinal maturation in the early postnatal life may be an important factor in the protective effects of SL intervention on early weaning diarrhea in neonatal piglets.

Although the precise molecular and cellular mechanisms of how SL intervention upregulates GDNF expression and activates the downstream protein kinase-mediated phosphorylation of CREB remains unresolved, it is possible that the Sia moiety of SL is a critical player for the activation of both the GDNF and polySia-NCAM signaling pathways. This hypothesis is supported by our present and previous studies, including the following: (1) Sia is an essential conditional nutrient for neural development and cognitive function for the newborn [43] and also for proper neuron function in the ENS in the elderly [49]; (2) Sia is a key sugar component of sialylated milk oligosaccharides, glycoproteins, and gangliosides in human milk, where 69–82% of the Sia is conjugated as oligosaccharides [12]; (3) polysialylation of NCAM is involved in enteric precursor migration and neurite formation [50] and has been reported to mediate Ret-independent GDNF signaling [41]; (4) as shown in this study, expression levels of both GDNF and ST8Sia IV are upregulated by SL intervention. These findings imply that the interaction between GDNF and polySia-NCAM is associated with the beneficial effect

of SL intervention in promoting intestinal maturation in neonatal piglets. Our proposed molecular mechanism of how dietary SL intervention may influence the intestinal maturity and plasticity through a CREB dependent, polysialyltransferases, and polySia-NCAM-mediates binding to GDNF promoting cell proliferation is summarized schematically in Fig. 7.

In summary, the collective results of our current study demonstrate that SL intervention can promote gut development and prevent early weaning diarrhea in neonatal piglets by upregulating the transcriptional and translational levels of GDNF, CREB phosphorylation, Ki67, and ST8Sia IV expression in the ileum of a piglet. Given the emerging importance of the role of the "gut–brain axis" and ENS in intestinal health and disease prevention, and the impact of SL intervention and Sia on CNS and ENS, future studies will be required to assess the molecular underpinnings of more precisely how SL intervention may impact the "gut–brain axis" during development. Finally, it is noted that these findings add further proof that the neonatal piglet model is very informative in understanding the molecular mechanisms regulating the efficacy of dietary interventional studies on intestinal maturity, neurocognition, learning, and memory for translational value to humans, in contrast to the mouse model.

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Compliance with Ethical Standards

The experimental protocol was carried out in accordance with guidelines established by the National Natural Science Foundation of China and approved by the Animal Ethics Committee of Xiamen University.

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

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