Original Article

Enhancement of motor neuron development and function in zebrafish by sialyllacto-N-tetraose b

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Background: Sialyllacto-N-tetraose b (LSTb) is a component of human milk oligosaccharides. Due to its low concentration, the impact of LSTb on neurodevelopment remains largely unexplored. It is worth studying whether LSTb should be added to infant formula to simulate breast milk. This study aimed to investigate the effect of LSTb on the development of motor neurons of the central nervous system using a transgenic zebrafish model.

Methods: Transgenic (Tg) zebrafish line (Hb9:GFP) was incubated with LSTb, and the axonal growth of caudal primary (CaP) neurons was assessed. Locomotor behavior was evaluated, and RNA sequencing (RNA-seq) was performed to identify the differentially expressed genes (DEGs). The expression of *Slit2* and *Slit3*, genes involved in axon guidance, was further analyzed through real-time polymerase chain reaction (real-time PCR) and whole-mount *in situ* hybridization.

Results: There was a significant increase in the number and length of CaP axon branches, suggesting that LSTb promotes CaP development. Behavioral analysis revealed enhanced locomotor activity in LSTb-treated larvae, indicating improved motor function. RNA-seq analysis identified 5,847 DEGs related to central nervous system neuron differentiation, including *Slit2* and *Slit3*, which are known to contribute to axon guidance. In situ hybridization confirmed increased *Slit2* expression in the central nervous system of LSTb-treated larvae.

Conclusions: LSTb significantly influences motor neuron development, potentially through the upregulation of *Slit2* and *Slit3*. This research provides valuable insights into the role of LSTb in neurodevelopment.

Keywords: Sialyllacto-N-tetraose b (LSTb); zebrafish; caudal primary neurons (CaP neurons); *Slit2*; *Slit3*

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Introduction

Human milk oligosaccharides (HMOs) are a type of polysaccharide and the third largest component of human milk after lactose and lipids (1). They serve as prebiotics and play a significant role in brain development and in programming the immune system to reduce the incidence of allergies and autoimmune diseases (2,3). More than 200 different structures of HMOs have been identified in human milk. They primarily consist of monosaccharides such as D-glucose (Glc), D-galactose

(Gal), *N*-acetyl-D-glucosamine (GlcNAc), L-fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) (4), which form a series of complex oligosaccharides that can be classified into three major types based on their fucosylation or sialylation: (I) neutral nonfucosylated HMOs; (II) neutral fucosylated HMOs; and (III) HMOs containing sialic acid (5,6).

Sialyllacto-N-tetraose b (LSTb) is a type of sialylated oligosaccharide found in mammal milk. It comprises three monosaccharide units, including galactose, glucose, and lactose. There are individual differences in the concentrations and distributions of LSTb in breast milk (7,8). However, due to the low concentration of LSTb in milk, research on its effects is limited. Whether LSTb, similar to other HMOs, affects the development of the central nervous system is still poorly understood.

Zebrafish have gained recognition as a popular model organism in neurobiology in recent years (9). Both adult and larval zebrafish have greatly contributed to neuroscience due to their remarkable physiological and genetic similarities to humans (10,11), as well as their amenability to gene manipulation and comparable central nervous system morphology (12). Zebrafish provide a stable platform that balances the intricacy of a vertebrate and its utility as a model organism. With the added benefits of optical transparency in developing fish facilitating the application of advanced imaging techniques, these complex mechanisms at the organism level can be effectively visualized (13).

This study used the transgenic (Tg) zebrafish line (Hb9:GFP), which is commonly used to evaluate motor neurons of the central nervous system. We incubated the zebrafish with LSTb to gain insight into its influence on nervous system development. We present this article in accordance with the MDAR and ARRIVE reporting checklists (available at https://tp.amegroups.com/article/view/10.21037/tp-24-247/rc).

Highlight box

Key findings

- Sialyllacto-N-tetraose b (LSTb) significantly enhances the development and functionality of motor neurons in zebrafish through the upregulation of Slit2 and Slit3.

What is known and what is new?

- Human milk oligosaccharides (HMOs) are widely acknowledged to be essential for brain development and immune system programming, and LSTb is a low-concentration component of HMOs.
- LSTb substantially impacts the development and function of motor neurons in zebrafish.

What is the implication, and what should change now?

- This study's findings suggest that LSTb may have a broader effect on neurodevelopment, potentially influencing the growth and guidance of neurons beyond the motor system.
- The upregulation of *Slit2* and *Slit3* indicates a possible mechanism by which LSTb exerts its effects, which could be targeted for therapeutic interventions in neurodevelopmental disorders.
- Further research should focus on the effects of LSTb in other animal models and eventually in human clinical trials to explore its potential as a therapeutic agent for neurodevelopmental disorders.
- The findings also underscore the importance of including LSTb in the formulation of infant formulas to potentially mimic the benefits of breast milk and support optimal brain development.

Methods

Zebrafish husbandry

Specimens of the Tg zebrafish line (Hb9:GFP) were acquired from the Zebrafish Facility of Nantong University. Experiments were performed under a project license (No. S20210310-007) granted by the ethics committee of Nantong University, in compliance with institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration. The zebrafish larvae were kept in a recirculation system under a 14-hour light and 10-hour dark photocycle at 28±0.5 ℃ and a pH range of 7.0–7.5. Embryos at 8 hours post-fertilization (hpf) were co-incubated with 1 mg/mL of LSTb (DSM, Maastricht, the Netherlands) as the LSTb group. The control group was incubated according to the normal process.

Image observation of Tg zebrafish

Zebrafish larvae were placed in 0.7% low-melting-point agarose. Confocal imaging was carried out at 72 hpf. Embryos were anesthetized with egg water and 0.16 mg/mL of tricaine to acquire good pictures. Confocal imaging was accomplished using the TCS-SP5 laser scanning microscope (Leica, Wetzlar, Germany) and analyzed using Imaris (Oxfords Instruments, Abingdon, UK) and ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

Zebrafish larva locomotor behavior test

In accordance with previously established methods (14), 30 zebrafish larvae at 5 days post-fertilization (dpf) were

selected at random from groups and plated individually within each well of a 24-well plate. The DanioVision Observation Chamber (Noldus, Wageningen, the Netherlands) was employed to facilitate the measurement and observation of the swimming behavior of the larvae. Prior to monitoring, the larvae were allowed to acclimate for a period of 30 min at 28 ℃. The Ethovision XT 13 system (Noldus) was then used to complete the locomotor behavior test of the zebrafish larvae within 30 min. The software computed the locomotor behavior distance and spontaneous movement, with the average speed being derived for each group.

RNA sequencing (RNA-seq) analysis

We performed RNA-seq at 72 hpf. Total RNA from zebrafish was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed into complement DNA (cDNA) using the first strand of transcriptional cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). Library sequencing was performed using a HiSeq 4000 platform (Illumina, San Diego, CA, USA). Raw readings were filtered to exclude low-quality data, and high-quality raw data were used for downstream analyses. Differential expression analysis was completed with the DESeq2 (v. 1.6.3) R package. Differential gene expression changes of more than two-fold and q value ≤0.05 indicated a significant difference. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed genes (DEGs) were completed.

Real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted using TRIzol UP (TransGen Biotech, Beijing, China) and transcribed into cDNA using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). The primer used was *Slit2* and *Slit3* (*Slit2* forward primer: GCTGTTCAGGCG TAGAAGACTACC; *Slit2* reverse primer: GTATACTGAGGGATGTGGTCTGGGA; *Slit3* forward primer: TCCCAACTTGTCCTACCTGTAGTGT; *Slit3* reverse primer: ACCCACCGCATCATATATCCTCTGA).

Whole-mount in situ hybridization

The cDNA served as a template for cloning fragments of *Slit2* to create antisense RNA probes for zebrafish *Slit2* and *Slit3* at 24, 48, and 72 hpf. The *Slit2* primers were determined via reverse transcription PCR (RT-PCR). Digoxigenin (DIG)-labeled RNA sense and antisense probes were generated from the linearized plasmids with a DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics). Whole-mount *in situ* hybridization was conducted as per previously reported procedures (15).

Measurement and statistical analysis

The axonal number and length of caudal primary (CaP) neurons were measured using ImageJ software. The branch numbers within the distal 50 μm and axonal length were calculated. All data analysis and statistical comparisons were completed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined using the Student's *t*-test. Data are presented as the mean ± standard deviation, and P values <0.05 were considered to be statistically significant.

Results

Effects of LSTb on the axonal growth of CaP neurons

LSTb treatment significantly increased the number of CaP axon branches per 50 μm at 72 hpf compared with that observed in the control group (18.05±0.8568 *vs.* 12.00±0.5282) (*Figure 1A*,*1B*). The average length of the CaP axon in the LSTb group (229.4±4.219 μm) was significantly longer than that of the control group (183.9±2.119 μm) (*Figure 1A*,*1C*).

Locomotor behavioral changes induced by LSTb

As shown in *Figure 2A*, zebrafish larvae of the LSTb group exhibited more frequent behavioral trajectories at 5 dpf. In the LSTb group, the distance and speed of the zebrafish larvae notably increased (*Figure 2B*,*2C*).

Transcriptomic profiling of LSTb treatment

We identified 5,847 DEGs between the two groups, with 2,201 genes being upregulated and 3,646 genes being downregulated (*Figure 3A*). According to GO annotations, central nervous system neuron differentiation was noted as the representative GO term enriched from DEGs in biological processes (BP) (*Figure 3B*). We also observed 33 upregulated DEGs in GO term of central nervous system

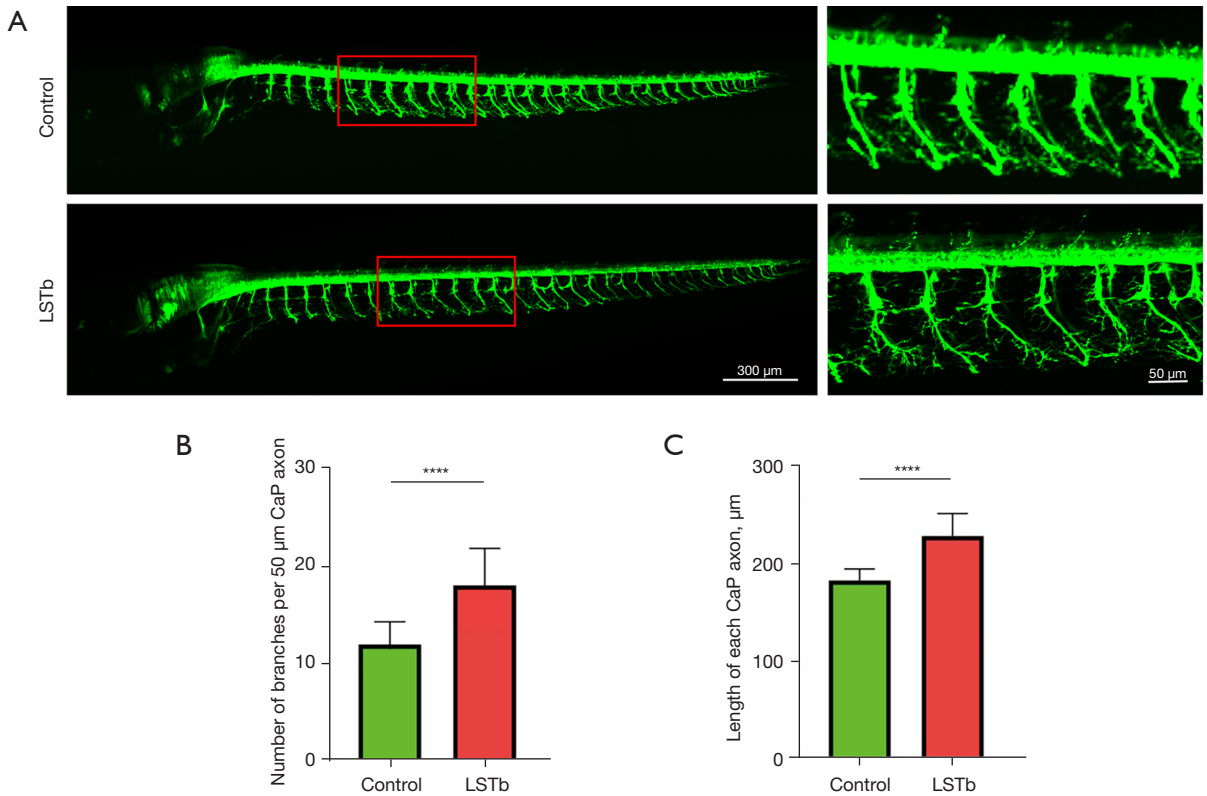

Figure 1 Effects of LSTb on motor neurons in Tg zebrafish (Hb9:GFP). (A) Fluorescence images of the motor neuronal axons of Tg zebrafish (Hb9:GFP). The right images of the control group and LSTb group are partial enlargement of the red box in the left image. (B,C) Statistical analysis of number of branches and length of CaP. n=30 in each group. ****, P<0.0001 compared to control group. LSTb, sialyllacto-N-tetraose b; CaP, caudal primary; Tg, transgenic.

neuron differentiation. Among these 33 DEGs, *Slit2* and *Slit3* are classic genes for axon guidance (*Figure 3C*). To verify the reliability of RNA-seq, we conducted quantitative RT-PCR on the expression of *Slit2* and *Slit3* at 72 hpf. The expression of *Slit2* and *Slit3* was consistent with the that from the RNA-seq results, with significantly increased expression in the LSTb group (*Figure 3D*).

Expression of Slit2 in zebrafish

We analyzed the temporal and spatial expression patterns of *Slit2* in embryos at 24, 48, and 72 hpf. *Slit2* was ubiquitously expressed in the central nervous system, including the brain and spinal cord. Notably, *Slit2* expression was more enhanced in the LSTb group compared with that in the control group after fertilization (*Figure 4*). This suggested that LSTb could promote the expression of *Slit2* in the central nervous system.

Discussion

Sialylated HMOs (SHMOs) are a type of HMO that constitute about 20% of all HMOs (16,17). SHMOs play an important role in the growth and development of the brain and nervous system in infants (18,19) and serve as a source of nutrients for infant neurodevelopment. LSTb is a specific type of SHMO (molecular formula: C37H62N2O29; molecular weight: 998.9 g/mol) and consists of one lactosamine Galβ1-3/4GlcNAc, one lactose core Galβ1-4Glc, and one Neu5Ac. The structural sequence of LSTb is Galβ1-3 (Neu5Acα2-6) GlcNAcβ1-3Galβ1-4Glc (Neu5Ac 1 Gal 2 Glc 2).

The levels of LSTb in human breast milk are relatively low and can be affected by various factors. This includes regional differences, as shown by the modest LSTb level of 41±5 nmol/mL seen in Peruvian women (7). LSTb concentrations typically peak during lactation days 8 to 10

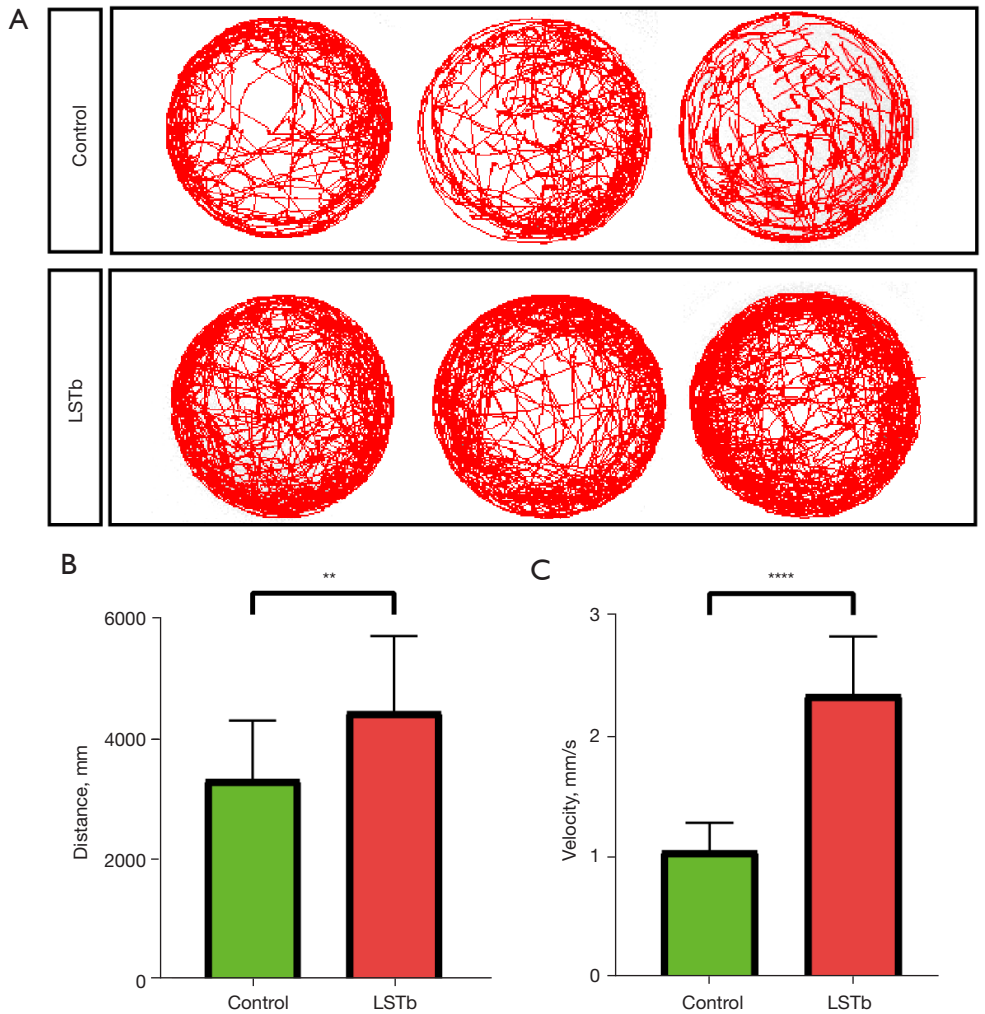

Figure 2 Neurobehavioral changes induced by LSTb. (A) Representative images of locomotor tracks. (B) Distance. (C) Average velocity. n=30 in each group. **, P<0.01; ****, P<0.0001, compared to the control group. LSTb, sialyllacto-N-tetraose b.

and then decrease (10.3390/nu15061408). Additionally, a negative correlation was found between maternal body mass index at 5 months and LSTb level (20). A study on the relationship between LSTb and infants revealed that LSTb levels were negatively associated with weight and head growth at 14 and 28 days postpartum (21). LSTb levels at 6 months were also demonstrated to be predictive of cognitive development scores in infants (22). It is possible that LSTb levels could be linked to infant cognitive development through the proliferation or depletion of specific gut microbes (23,24).

The impact of LSTb on cognitive performance in infants differs from that of other HMO constituents. To investigate the effects of LSTb on central nervous system development, we opted to use Tg zebrafish and study motor neuron development. The results indicated that coincubation of zebrafish with LSTb increased both the number and length of CaP neuron branches, suggesting that LSTb indeed facilitates the growth of CaP neurons. CaP, a type of primary motor neuron, is closely related to locomotion. Thus, we conducted behavioral testing, which showed that immersion of zebrafish larvae in LSTb resulted in notable enhancements in swimming distance, tail-wagging movement, and average speed. RNA-seq was performed to understand how LSTb promotes CaP development, and the results showed that zebrafish larvae soaked in LSTb exhibited increased expression in 33 genes, with GO entries enriched in central nervous system neuron differentiation.

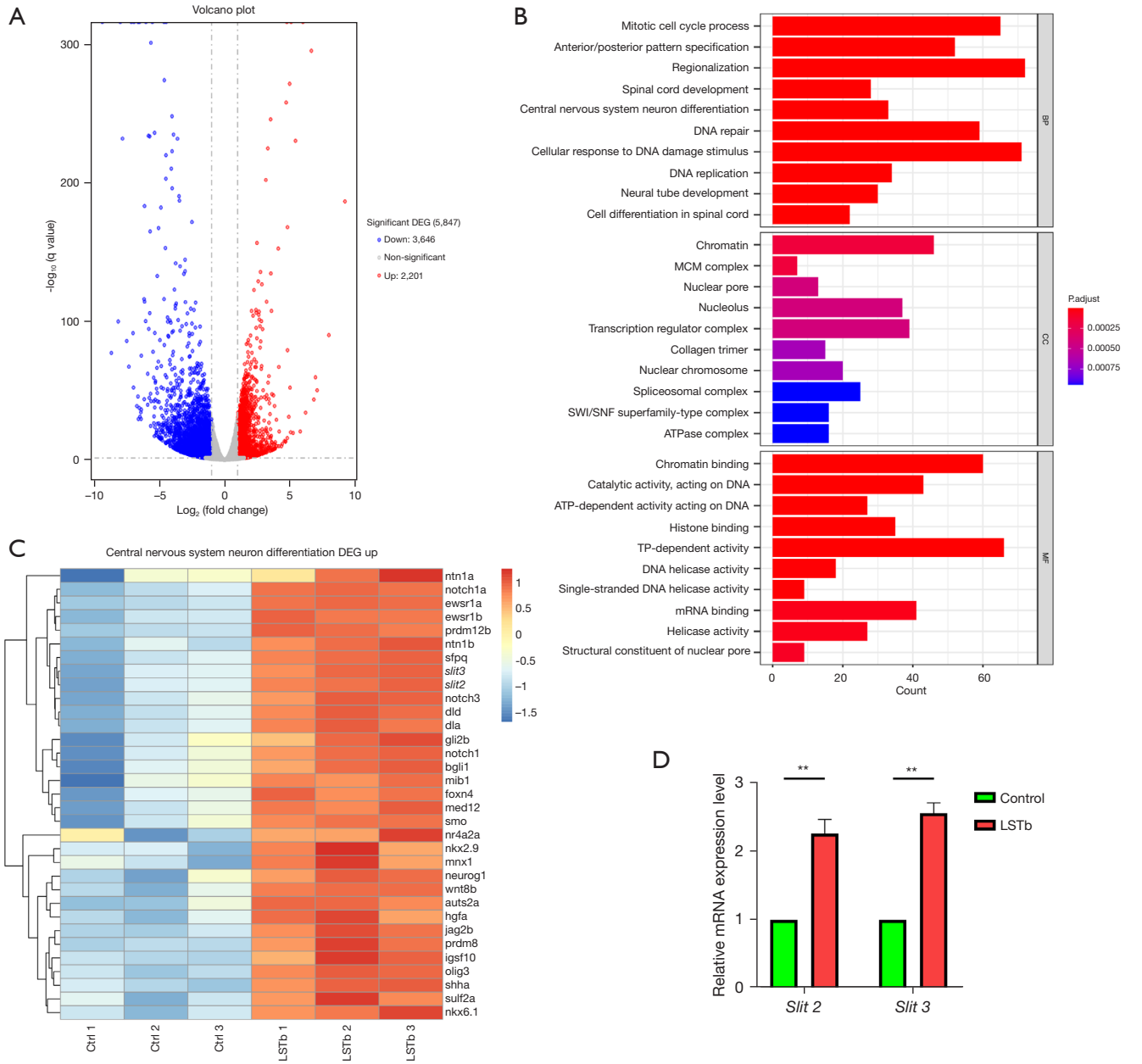

Figure 3 Transcriptomics profile of zebrafish in the LSTb group. (A) Volcano map of DEGs. Red spots represent upregulated genes, and blue spots represent downregulated genes. (B) GO annotation of DEGs. (C) Heatmap genes showing upregulation of central nervous system neuron differentiation in the LSTb group versus the control group. Red indicates high correlation and blue indicates low correlation. (D) Expression of *Slit2* and *Slit3* between the LSTb and control group according to real-time PCR (30 larvae pooled as one sample; n=3). **, P<0.01. DEG, differentially expressed gene; BP, biological process; CC, cellular component; MF, molecular function; MCM, minichromosome maintenance; SWI/SNF, switch/sucrose non-fermenting; LSTb, sialyllacto-N-tetraose b; ATP, adenosine triphosphate; GO, Gene Ontology; RT-PCR, reverse transcription polymerase chain reaction.

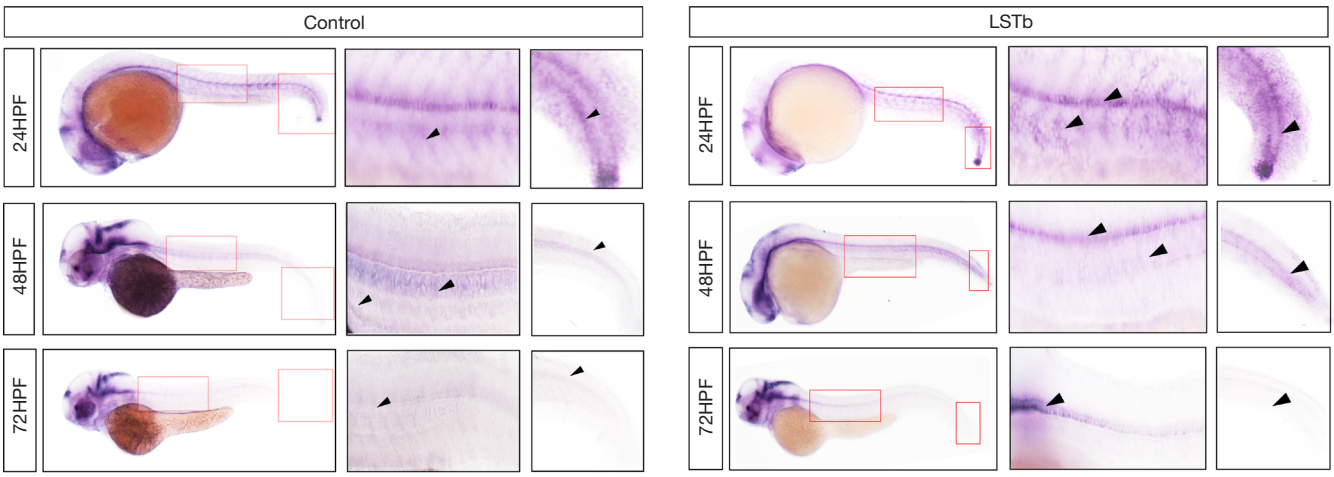

Figure 4 *Slit2* expression of larvae in the LSTb and control groups at 24, 48, and 72 hpf according to whole-mount in situ hybridization. Enlarged images, with the positive signals being marked with black arrowheads. hpf, hours post-fertilization; LSTb, sialyllacto-N-tetraose b.

Slit2 and *Slit3*, classic genes related to axon guidance became the focus of our attention. Subsequent verification by PCR also showed that the expression of *Slit2* and *Slit3* increased significantly after LSTb treatment. The whole-mount *in situ* hybridization results also showed that the expression of *Slit2* at different times after incubation with LSTb was stronger than that in the control group. Based on these findings, we speculate that LSTb promotes CaP neuron development through *Slit2* and *Slit3*.

The Slit family is a group of secreted proteins that hold critical significance in guiding neuronal axons. Slit proteins consist of single peptides that contain about 1,500 amino acids. In vertebrates, the Slit family includes *Slit1*, *Slit2*, and *Slit3* (25). In zebrafish, four genes have been characterized, namely *Slit1a*, *Slit1b*, *Slit2*, and *Slit3* (26,27). Among the Slit proteins, *Slit1* is mainly expressed within the developing nervous system, while *Slit2* and *Slit3* are present in other parts of the body, such as the kidney, lungs, heart and, immune cells, in addition to the nervous system (28-30). A study conducted in the late 1990s showed that *Slit2* within the calf brain led to a fivefold increase in the number of branch points per axon and to 2.5-fold increase in axon length (31). Slit has been identified as a positive regulator of dorsal root ganglion axonal branching and elongation (32). Slits typically exert their biological function by binding to roundabout (Robo) receptors, and Slit-Robo signaling plays a role in various BP, including axon guidance and central nervous system development (33).

There are some limitations to this study that should be mentioned. Although the study examined the changes of structure of CaP neurons and motor ability, the effect of LSTb on motor neuron differentiation was not observed, and other types of neurons in the central nervous system and their corresponding functions were not taken into account. Additionally, we used zebrafish and did not investigate the higher functions of the central nervous system, such as learning and memory. Our findings indicated that LSTb, despite not being a major component of breast milk, can significantly impact the development of motor neurons. The mechanism behind this effect may be related to the increase in the expression of *Slit2* and *Slit3* by LSTb. In order to further explore the impact of LSTb on the central nervous system, we plan to conduct more research using mammalian models in the future, providing more reliable experimental data and theoretical support for the addition of LSTb to infant formula.

Conclusions

The study provides evidence that LSTb, despite its low concentration in human milk, has a significant impact on the development and function of motor neurons in zebrafish. LSTb may modulate neurodevelopment by regulating the *Slit2* and *Slit3* genes that are critical for axonal growth and guidance. These findings contribute to our understanding of the role of HMOs in neurodevelopment and provide new avenues for further exploration of LSTb's potential as a therapeutic agent for neurodevelopmental disorders.

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Footnote

Reporting Checklist: The authors have completed the MDAR and ARRIVE reporting checklists. Available at https://tp.amegroups.com/article/view/10.21037/tp-24-247/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-247/dss

Peer Review File: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-247/prf

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. S20210310-007) granted by the ethics committee of Nantong University, in compliance with institutional guidelines for the care and use of animals.

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