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RESEARCH ARTICLE

Vagus Nerve Stimulation Improves Cognitive Impairment After Traumatic Brain Injury Through the "Microbiota-Gut Immune Barrier-Neuroinflammation" Axis

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ABSTRACT

Objective: Traumatic brain injury (TBI) often leads to physical and mental dysfunction in human and companion animal. Studies have shown that the gut microbiota influences TBI progression and prognosis through the gut-brain axis. Vagus nerve stimulation (VNS) has neuroprotective and cognitive benefits, but its mechanism for alleviating cognitive impairment after TBI remains unclear. Methods: A TBI model was established using a controlled cortical impact device (CCI), with 3-day VNS intervention. To investigate the relationship between VNS improving cognitive impairment after TBI and remodeling gut dysbiosis, antibiotics were used to clear the gut microbiota of TBI mice. Cognitive improvement was evaluated via behavioral tests. Inflammation, gut microbiota, and colonic damage were analyzed via Nissl staining, immunofluorescence, Hematoxylin-eosin staining, ELISA, 16S rRNA gene sequencing, qRT-PCR, and western blot. Results: Our study revealed that VNS markedly enhanced cognition and inhibits neuroinflammation in TBI mice by modulating the TLR4/MyD88/NF-κB pathway, while simultaneously reducing the abundance of cognition-impairing associated *Escherichia_Shigella* and ameliorating intestinal barrier dysfunction. After antibiotics eliminated the microbe, the effects of VNS on improving cognition, alleviating colon injury, and suppressing neuroinflammation were weakened. Conclusions: These findings reveal a novel microbiota-gut immune barrier-neuroinflammation axis, supporting VNS-based interventions for TBI management in both humans and companion animals.

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INTRODUCTION

Traumatic brain injury (TBI) has emerged as a leading threat to human and companion animal health and life (Finnie, 2012). With the development of modern industry, power machinery, and high-speed transportation, the incidence of TBI is increasing (Finnie, 2014). Due to the high morbidity, mortality, disability rate, and health care costs associated with TBI, they not only pose a serious threat to people's safety but also impose a substantial economic burden on the world today (DiFazio and Fletcher, 2013). Cognitive impairment is the most common and longest-lasting neurological complication of secondary injury after TBI (Kuo *et al.*, 2018). Options currently available for the management of cognitive impairment after TBI include cognitive rehabilitation, medical therapy, and non-invasive brain stimulation (NBS), but the efficacy remains suboptimal (Robba *et al.*, 2025). Thus, it is urgent that a solution be found to improve cognitive impairment to minimize adverse effects on TBI patients and animals.

The microbiota-gut-brain axis has sparked a research surge in academia recently. It has been reported that TBI can lead to intestinal mucosal injury, and increased intestinal permeability, and changes in the microbiota can affect the prognosis of TBI (Verdoodt *et al.*, 2022). As a result, the gut microbiome could be considered for therapeutic intervention in TBI (El Baassiri *et al.*, 2024). Clinical studies confirmed the presence of microbiota changes during the chronic phase in patients with TBI (Hou *et al.*, 2021). Within 2 hours following moderate TBI, the gut microbiota in rats altered, showing a reduction in beneficial bacteria and a marked rise in pathogenic bacteria (Nicholson *et al.*, 2019). TBI disrupts the balance of gut microbiota, impairing intestinal barrier integrity and

immune regulation. This dysfunction facilitates the translocation of bacteria, endotoxins, and pro-inflammatory mediators into the circulatory system. This induces a systemic inflammatory response, which then results in inflammation within the central nervous system (Solanki *et al.*, 2023). In contrast, neuroinflammation was reduced after restoration of intestinal microflora by transplanting fecal microbiota (Hu *et al.*, 2023). Therefore, the "microbiota-gut immune barrier-neuroinflammation" axis may be an effective way to block the development of cognitive impairment after TBI.

Vagus nerve stimulation (VNS) has anti-inflammatory properties that benefit various diseases (Dawson *et al.*, 2021). The use of VNS has been extended to cover more brain disorders like Parkinson's, Alzheimer's, stroke, and TBI (Kumaria and Tolias, 2021). Zhang et al. found that VNS alleviated cognitive impairment after TBI by reducing oxidative stress and inflammatory responses, strengthening the blood-brain barrier's integrity, and managing neurotransmitter activity (Zhang *et al.*, 2022). Recent findings have shown that VN activation presents anti-inflammatory effects and has a positive impact on the intestinal microflora and the production of beneficial bacteria (Faraji *et al.*, 2025). However, it remains unclear whether the VNS improves cognitive impairment after TBI by affecting the intestinal microbiota and neuroinflammation.

The present study aimed to investigate the effect of VNS on cognitive impairment in mice with TBI. Our findings demonstrated that VNS improved cognitive impairment in TBI mice and reduced neuroinflammation by remodeling intestinal microbiota and improving the gut immune barrier. This investigation implies that VNS improves cognitive deficits from TBI, potentially due to its positive impact on gut microbiota and neuroinflammation.

MATERIALS AND METHODS

Animals and TBI model: Male C57BL/6N mice (SPF grade, 8-10 weeks of age, 22-24 g body weight) were procured through **** University Experimental Animal Center (**, China). Subjects underwent 7-day acclimatization under standardized vivarium conditions (ambient temperature 24±1°C, relative humidity 45±5%, 12h:12h light-dark cycle) prior to experimental interventions. All experimental procedures complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanchang University.

A controlled cortical impact device was used to establish a TBI model in mice (Yu *et al.*, 2025). The mice were anesthetized by inhaling a mixture of air/oxygen (40% oxygen: 60% nitrogen) containing 3.5% volume sevoflurane through a mask, while receiving subcutaneous injection of butorphanol for analgesia (0.1 mg/kg). The mice were fixed into an impact device PSI-0310 stereotaxic frame. A 3.0 mm burr hole was drilled through the right parietal bone (AP: +2.0 mm from bregma, ML: ±1.0 mm) using an orthopedic drill system. There was an impact parameter of 8 m/s velocity, 150 ms contact duration, and 1 mm depth selected to create moderate to severe TBI. Immediately following the brain damage, the mice were sutured and kept warm until they awoke. Sutures were placed after craniotomies for sham surgery.

Experimental design and VNS treatment

This study included two independent experimental procedures: In Experiment 1, to assess VNS efficacy against cognitive deficits in post-TBI mice, 48 mice were assigned randomly into three cohorts: Control group, TBI group, TBI+VNS group (n=16/group). The mice were subjected to auricular vagus nerve stimulation. Before the experiment, mice were lightly anesthetized with isoflurane by inhalation, and the stimulating electrode was placed in the left concha area (Lv *et al.*, 2024). The wires were connected to an IKing-IV type monitor. Stimulation parameters: 1.0 mA intensity, 0.5 ms pulse width, 20 Hz frequency, 30 min per day, lasting for 3 days. The control and TBI mice underwent similar treatments as the TBI+VNS mice but without electrical stimulation. We randomly recorded survival rate of 8 mice in each group.

In Experiment 2, to verify whether VNS improves cognitive impairment after TBI by regulating gut microbiota, 32 mice were assigned randomly into four cohorts: Control group, TBI group, TBI+VNS group, TBI+VNS+antibiotic group (n=8/group). In the TBI+VNS+antibiotic group, a mixture of antibiotics (a combination of 5 mg/mL neomycin sulfate, 1.25 μg/mL natamycin, and 5 mg/mL bacitracin) was used as the daily drinking water for mice, continuously fed for 7 days to eliminate gut microbiota, and then intervened with VNS for 3 days.

Assessment of neurological injury: According to the methods described in the literature, neurological function was measured using the Longa neurological score after the intervention (Zhong *et al.*, 2022). The total score was 4 points, with a higher score indicating more severe nerve damage in mice.

Behavioral assessment: According to the methods described in the literature, Morris water maze (MWM) and open field test (OFT) were performed on mice to assess their cognitive function (Gu *et al.*, 2024).

Measurement of survival rate, body weight, and brain weight: The survival rate was monitored for 15 months post-treatment. To assess the impact of VNS and TBI on brain tissue, mice's body and brain weights were recorded for calculating the brain-to-body weight ratio.

Nissl staining: According to the methods described in the literature, Nissl staining was used to observe neuropathological changes in hippocampal tissue (Zhi *et al.*, 2024).

Immunofluorescence: Immunofluorescence was conducted according to the method described earlier (Cheng *et al.*, 2025). The immunodetection system employed three rabbit-derived primary antibodies targeting c-Fos (1:500), bromodeoxyuridine (BrdU, 1:500), and glial fibrillary acidic protein (GFAP, 1:500), with visualization achieved using Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:1000). All antibodies

were purchased from Invitrogen, USA. The nuclei of cells were counterstained with Hoechst (Sigma, USA) for 10 minutes.

Hematoxylin-eosin staining (H&E): After initial fixation with 4% paraformaldehyde, the colon specimens were processed through paraffin embedding and microtome-sectioned to obtain 5 μm thick sections for analysis. Following H&E staining, these tissue sections were carefully analyzed to identify pathological alterations in the colon structure.

ELISA: Following the manufacturer's protocol, the concentrations of TNF-α, IL-1β, and IL-18 in hippocampal tissue samples, as well as the levels of DAO, LPS, TNF-α, and IL-10 in mouse serum, were quantitatively analyzed using ELISA.

Quantitative real-time PCR (qRT-PCR) for RNA Expression Detection: qRT-PCR was conducted using a previously validated protocol (Liu *et al.*, 2024). The total RNA of mouse hippocampus and colon tissues was extracted using TRIzol reagent (Accurate Biotechnology, China). RNA was reverse-transcribed using a reverse transcription kit (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. The reverse transcription products were analyzed by SYBR Green Realtime PCR Master Mix. The primers used to determine relative gene expression are shown in Table 1. Expression levels were calibrated to β-actin, and each sample underwent three distinct amplifications.

Table 1: Primer sequences utilized for qRT-PCR

Target	Forward Primer (5'–3')	Reverse Primer (5'–3')
Cldn2	CGGGACTTCTACTCACCACTG	GGATGATTCCAGCTATCAGGGA
TjP1	CGGTCCTCTGAGCCTGTAAG	GGATCTACATGCGACGACAA
Ocln	GAGAGAGTGGACAGCCCCATTGCTGCTGTAATGAGGCTGC	
β-actin	CATGTACGTTGCTATCCAGGCCTCCTTAATGTCACGCACGAT	

Western blot analysis: Protein concentrations in hippocampal and colonic tissues were quantified using the BCA kit (Shanghai Biyun Tian Biotechnology Co., Ltd, Shanghai, China). The Western blot process was conducted in the manner previously described (Kang *et al.*, 2024). The following primary antibodies were utilized: mouse anti-β-actin (1:2000), rabbit anti-TLR4 (1:1000), rabbit anti-MyD88 (1:1000), rabbit anti-NF-κB p65 (1:1000), rabbit anti-Claudin-5 (1:500), rabbit anti-Occludin (1:500), and rabbit anti-ZO-1 (1:500). For detection, secondary antibodies consisting of goat anti-rabbit IgG and goat anti-mouse IgG were employed at a 1:5000 dilution. All antibodies were purchased from Abcam, USA. A chemiluminescence imaging system (Uvitec, Alliance Q9) was used to capture the protein bands. Each experiment was repeated three times.

Gut microbiota analysis: The murine fecal specimens underwent microbial genomic DNA extraction utilizing the QIAamp DNA Stool Mini Kit (QIAGEN, Germany). PCR amplification targeted the V3-V4 hypervariable regions of bacterial 16S ribosomal RNA genes, employing forward primer 338F (5'-ACTCCTACGGGAGGCAGC-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Amplicon sequencing was executed at Magi Gene Technology Company (Guangzhou, China) using the Illumina MiSeq sequencer (Illumina, USA). Subsequent bioinformatic processing of microbiota datasets employed QIIME2 software (version 2022.11) integrated with the R software package (version 3.2.0) for comprehensive metagenomic analysis.

Statistical analysis: The statistical evaluation was performed using SPSS Statistics (v26.0) and GraphPad Prism (v9.3.1). All experimental outcomes are expressed as mean ± standard deviation (SD). When comparing more than two experimental groups, Dunnett's post hoc test was applied following one-way analysis of variance (ANOVA). A p-value threshold of <0.05 was established to determine statistical significance.

RESULTS

VNS ameliorated cognitive impairment in TBI mice: The Longa score was conducted at post-injury day 3, with behavioral testing of animals performed from days 7 to 13 following TBI (Fig. 1A). To analyze the benefits of VNS on cognitive and neurological impairments in mice with TBI, we evaluated the Longa score and behavioral characteristics of mice after VNS. The TBI group had elevated Longa scores relative to the control group, but VNS treatment significantly reduced Longa scores, suggesting that VNS ameliorated TBI-induced neurological deficits (Fig. 1B). Body weight and brain weight were significantly reduced in TBI mice. VNS treatment effectively improved the body weight and brain weight of mice (Fig. 1C-D). The brain-to-body weight ratio did not differ significantly across the groups (Fig. 1E). We conducted the MWM test to examine the learning and memory functions of mice across the various groups. Control group mice exhibited short and direct trajectories, swimming directly towards the target quadrant, indicating normal spatial learning ability. In contrast, TBI group mice showed chaotic and circuitous wide-ranging search patterns, indicating impaired spatial memory. TBI+VNS group mice displayed significantly improved trajectories compared to the TBI group, characterized by shortened paths and a tendency to concentrate, suggesting that VNS intervention partially restored spatial memory capacity (Fig. 1F). TBI mice showed impairments in spatial learning and memory that rely on the hippocampus. VNS significantly improved memory impairment (Fig. 1G-J). We further observed whether VNS has an improvement effect on cognitive impairment in TBI mice through OFT experiment (Fig. 1K). The TBI group had a significantly shorter total distance travelled, no significant difference in the central motion distance, and a significantly longer time spent in the center. However, administration of VNS reversed this effect (Fig. 1L-N). In addition, we analysed the survival rates per cohort using Kaplan-Meier survival curves (Fig. 1O). Mice in the TBI and TBI+VNS groups began to die at months 2 and 4, whereas mice in the control group began to die at month 12. At the conclusion of the observation, mortality was 5% in the control group, 35% in the TBI+VNS and 65% in the TBI groups. These results indicate that VNS treatment significantly ameliorated TBI-induced cognitive impairment.

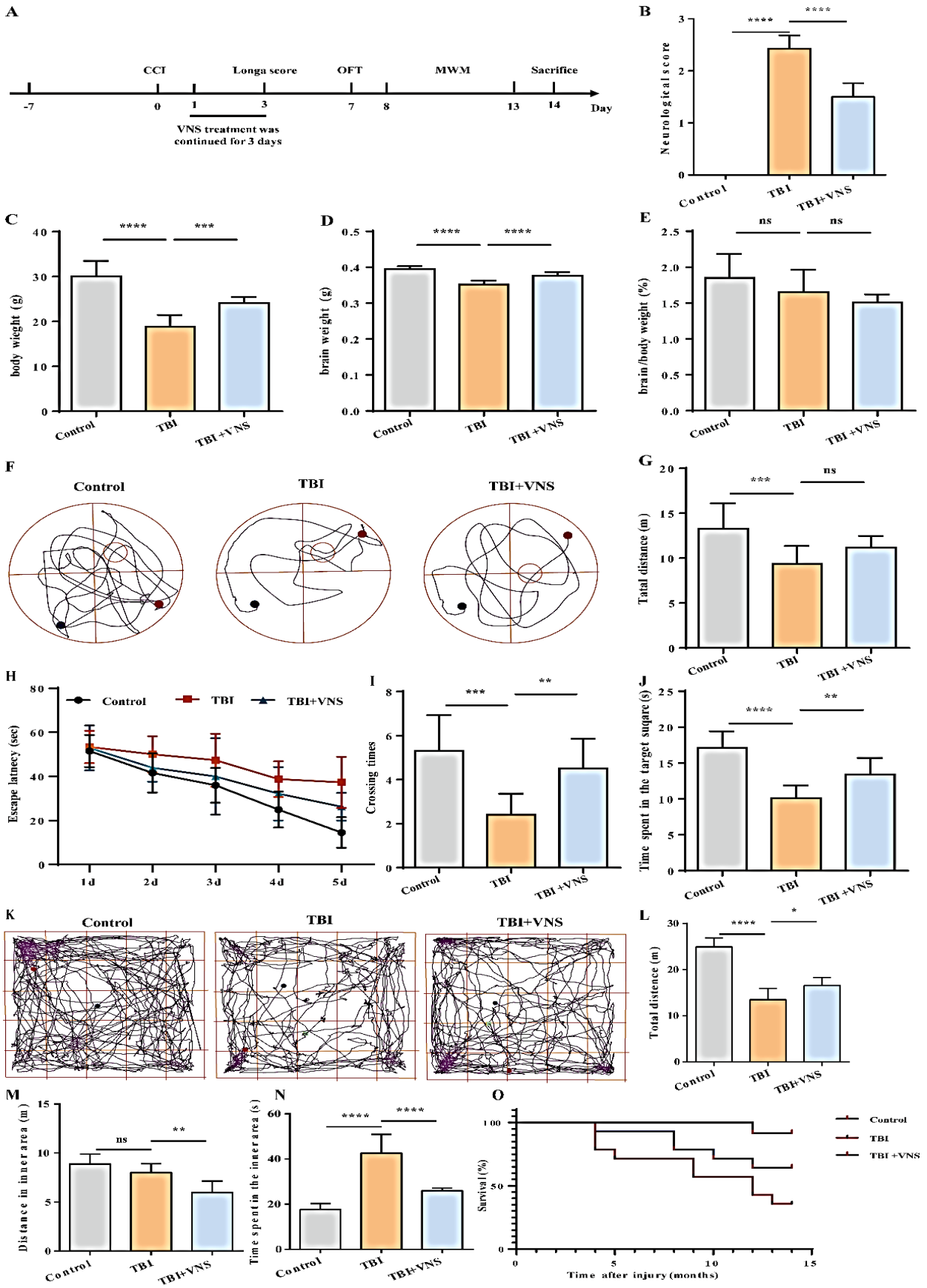

Fig. 1: VNS ameliorated cognitive impairment in TBI mice. (A) Timeline of the experimental procedure. (B) Neurological function of three groups was analyzed by Longa neurological score (n=8/group). (C-E) Quantitative analysis of body weight (C), brain weight (D), and brain-to-body weight ratio (E). (F) Representative trace chart of MWM test of the three groups (n=8/group). (G-J) Quantitative analysis of total distance (G), escape latency (H), times of crossing the platform (I), and duration in the target quadrant (J) in the MWM test. (K) Trace chart of mice in the OFT experiment (n=8/group). (L-N) Quantitative evaluation of total distance (L), central motion distance (M), and duration within the center (I) during the OFT experiment. (O) Kaplan-Meier survival curves were assessed for each group of mice 15 months after VNS (n=8/group). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Non-significant differences are represented by "ns".

VNS inhibited neuroinflammation in TBI mice: To analyze the impact of neuroinflammation on neurons, the hippocampal CA1 and CA3 were stained with Nissl to detect neuropathological alterations. In the control group, the hippocampal CA1 and CA3 had more neurons, with abundant Nissl bodies in the cytoplasm, and the neuronal cell shapes were rounded and arranged neatly. Following TBI, the neuronal cells of hippocampal CA1 and CA3 showed abnormal morphology, sparse and disordered arrangement, and a significant reduction or disappearance of Nissl bodies in the cytoplasm. However, VNS significantly improved these conditions (Fig. 2A-C). Immunofluorescence revealed alterations in the hippocampal density of c-Fos$^+$ cells (Fig. 2D). The TBI group showed a significant rise in c-Fos$^+$ cell density within the hippocampal granule cell layer (GCL), whereas VNS notably lowered this count (Fig. 2E). The effect of VNS on TBI-induced differentiation of neural progenitor cells to astrocytes was examined using dual immunofluorescent labeling, with BrdU$^+$ cells (red) identifying progenitors and GFAP$^+$ cells (green) marking astrocytes (Fig. 2F). In comparison to the control group, the molecular layer (ML) of the TBI group exhibited a marked increase in both BrdU$^+$ cell numbers and the (BrdU$^+$GFAP$^+$)/BrdU$^+$ ratio, whereas VNS significantly reduced this count, suggesting that VNS inhibited neural progenitor cell differentiation to astrocytes (Fig. 2G-H). Various neuroinflammatory responses, including significant increases in TNF-α, IL-1β, and IL-18, were observed in the hippocampus of TBI mice. VNS markedly reduced the concentrations of these proinflammatory cytokines (Fig. 2I-K). The TLR4 signaling cascade is considered an important therapeutic target for secondary brain injury after trauma (Hu *et al.*, 2022). We investigated TLR4, MyD88, and NF-κB p65 expression in the hippocampus to identify the molecular processes through which VNS counteracts neuroinflammation resulting from TBI. In TBI mice, the hippocampus showed a significant increase in TLR4, MyD88, and NF-κB p65 protein levels, which was mitigated by VNS (Fig. 2L-O). According to the data, VNS mitigated TBI-induced neuroinflammation through inhibition of the TLR4/MyD88/NF-κB signaling cascade.

VNS alleviated TBI-induced gut microbiota dysbiosis: To explore the relationship between intestinal microbial imbalance and post-TBI cognitive deterioration, we performed 16S rRNA gene sequencing to characterize temporal shifts in gut microbiome structure, revealing significant taxonomic variations correlated with memory impairment metrics. The analysis of α-diversity using Chao1 and Shannon indices indicated no notable difference in gut microbiota richness across the three groups (Fig. 3A-B). A notable difference in beta diversity was observed between the TBI and control groups; however, VNS treatment alleviated TBI-induced gut microbiota dysbiosis to some extent (PERMANOVA by Adonis, p=0.001) (Fig. 3C). Data analysis demonstrated a marked proliferation in the relative abundance of *Dubosiella*, *unclassified_Lachnos*P*iraceae*, *Muribaculum*, *28_4*, and *Bacteroides* in TBI mice, while *Allobaculum*, *Lactobacillus*, and *unclassified_Clostridia_UCG_014* significantly decreased in relative abundance (Fig. 3D). In addition, we further visualised the differential microbiota between the control and TBI groups by heatmap. The relative abundance of *Turicibacter*, *Dubosiella*, *Ileibacterium*, *Faecalibaculum*, *S*P*orosarcina,* and *Aerococcus* was marked reduction in the TBI group, whereas the relative abundance of *Enterobacteriaceae* significantly increased, suggesting that TBI may lead to gut microbiota dysbiosis (Fig. 3E).

It was previously confirmed that TBI can lead to cognitive impairment and gut microbiota disruption (Hanscom *et al.*, 2021). However, it is still unclear whether gut microbiota disruption is associated with cognitive impairment. We utilized the Mantel test to investigate the link between differential microbiota related to TBI and impairments in cognition and learning and memory. The richness of *Escherichia_Shigella* had significant positive correlations with the cognitive impairment and learning and memory ability ($P<0.05$) (Fig. 3F). Metabolic imbalance of L-Kynurenine as a core mediator of neuroinflammation and metabolic disorders after TBI is closely associated with secondary injury (Mani *et al.*, 2023). We further assessed the biological contribution of differential microbiota to L-kynurenine accumulation using random forest (RF) analysis and identified *Dubosiella*, *Alisti*P*es*, *lleibacterium*, *Candidatus_Saccharimonas*, *Allobaculum*, and *Muribaculum* as key predictors of L-kynurenine buildup in TBI mice ($R^2=0.8039$, $P<0.001$) (Fig. 3G). To determine the influence of VNS on *Escherichia_Shigella* in the colons of TBI mice, the abundance level of *Escherichia_Shigella* among the three groups was quantified. Consistent with previous results, our data revealed a notable elevation in *Escherichia_Shigella* levels within the TBI models, while VNS intervention exhibited potent inhibitory effects on gut microbiota proliferation in the colon (Fig. 3H). Overall, these results indicate that VNS alleviated cognitive impairment after TBI by regulating gut dysbiosis.

VNS protected intestinal barrier function in TBI mice: TBI-induced dysbiosis disrupts colonic mucosal barrier function (Chen *et al.*, 2025). Consequently, we explored the impact of VNS on the colonic tissue in mice with TBI. Histological analyses using H&E demonstrated that the control group had clear structures in all layers of the colon tissue, with well-organized intestinal glands and intact mucosa. Conversely, the TBI group's colon mucosa was incomplete, with some epithelial cell shedding, irregular arrangement of intestinal glands, widened spacing between intestinal glands, edema in the mucosal layer and submucosal layer, and minor infiltration of inflammatory cells. Compared with the TBI group, the TBI+VNS group exhibited reduced inflammatory cell infiltration, more regular glandular architecture, and improved epithelial cell arrangement (Fig. 4A). A colonic inflammatory response was triggered by TBI, marked by an increase in cytokines including DAO, LPS, TNF-α, and IL-10. VNS caused a significant reduction in DAO, LPS, TNF-α, and IL-10 levels (Fig. 4B-E). Cldn2, Tjp1, and Ocln, as core members of the tight junction protein family, play crucial roles in intestinal pathophysiology by regulating intercellular barrier function and signal transduction (Porter *et al.*, 2021). The mRNA level of Cldn2 was increased, while the expression of Tjp1 and Ocln was found to be reduced in TBI mice relative to the controls, indicating that TBI

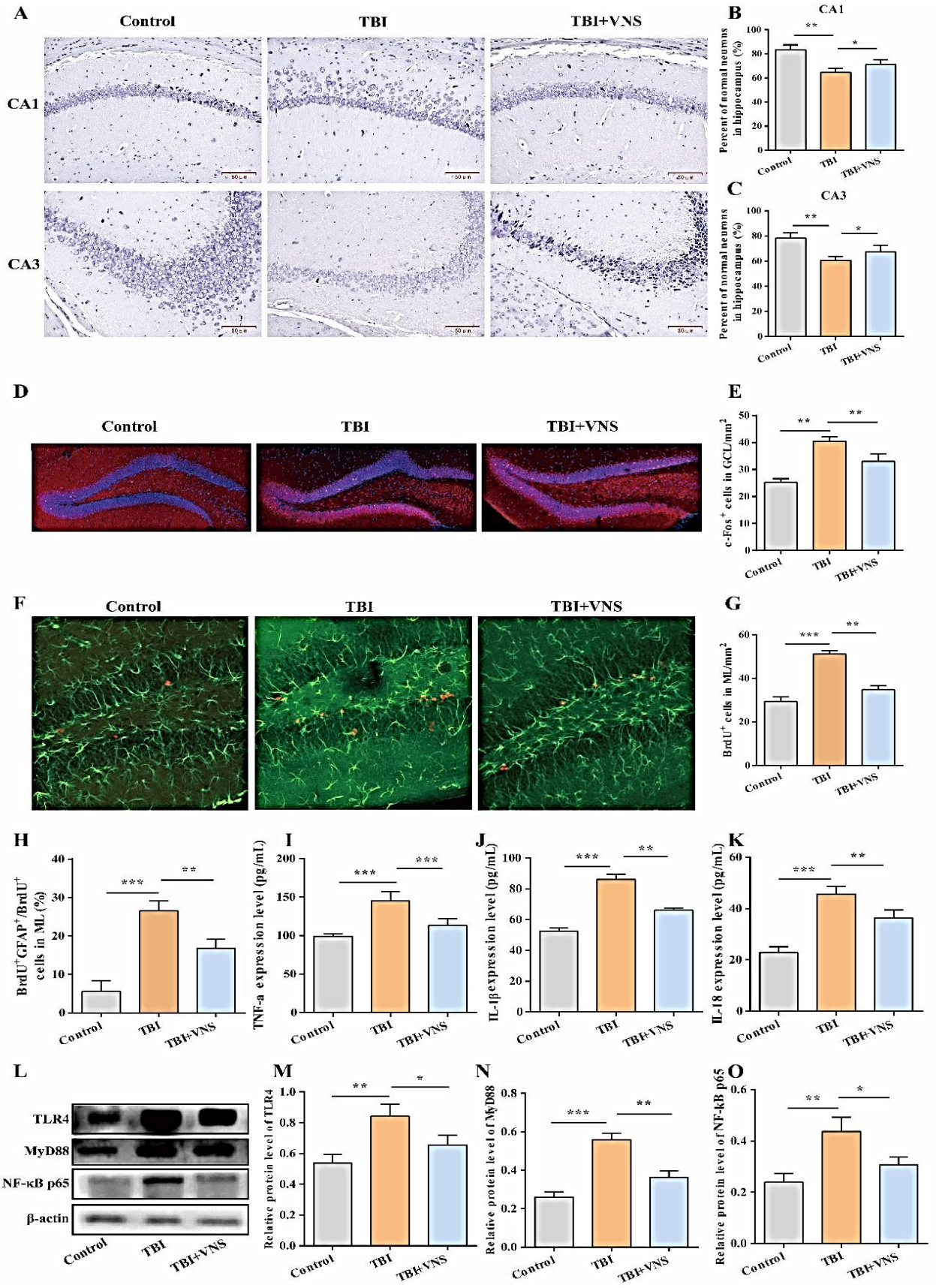

Fig. 2: VNS inhibited neuroinflammation in TBI mice. (A) Nissl staining of the hippocampal CA1 and CA3 in the control, TBI, TBI+VNS groups (n=8/group). Scale bar: 50 μm. (B-C) The percentage of normal neurons in the hippocampal CA1 (B) and CA3 (C) regions. (D) Confocal images illustrating immunolabeling of c-Fos (red) in the GCL. Hoechst (blue) was used to counterstain the nuclei. scale bars: 100 μm. (E) Statistical diagram of density of c-Fos$^+$ cells in the GCL. (F) Confocal micrographs illustrate dual immunofluorescence for BrdU (red) and GFAP (green) within the dentate gyrus. scale bars: 200 μm. (G) Statistical diagram of BrdU$^+$ cells density in the ML. (H) The ratio of (BrdU$^+$GFAP$^+$)/BrdU$^+$ in the ML. (I-K) The hippocampal expression of TNF-α (I), IL-1β (J), and IL-18 (K) was measured using ELISA. (L) Western blot bands representing TLR4, MyD88, and NF-κB p65 in the hippocampus. (M-O) Statistical examination of TLR4 (M), MyD88 (N), and NF-κB p65 (O) protein expression in hippocampal tissue. *P<0.05, **P<0.01, ***P<0.001.

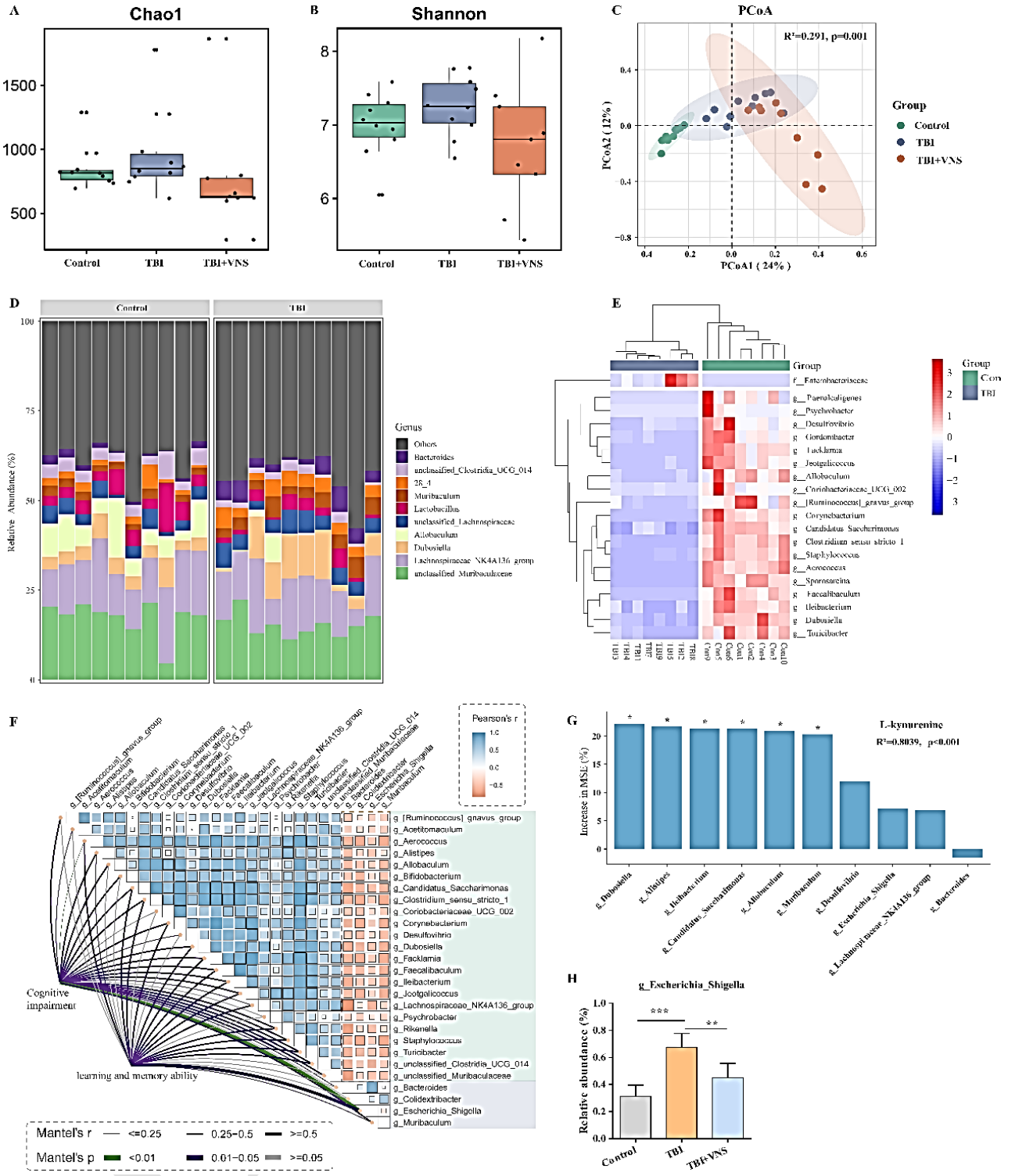

Fig. 3: VNS alleviated TBI-induced gut microbiota dysbiosis. (A-B) Chao1 (A) and Shannon (B) indices were used to analyze the α-diversity of gut microbiota (n=8/group). (C) PCoA plot showing gut bacteria compositional differences quantified by Bray–Curtis distance (PERMANOVA). (D) Bar diagram showing the abundance of bacteria at the genus level. (E) Heatmap showing the TBI-altered gut microbiota. (F) The relationship between TBI-associated changes in microbiota and cognitive impairment and learning and memory ability are shown, with a color gradient denoting Pearson's correlation coefficient. Edge thickness corresponds to the Mantel's r coefficient, which quantifies distance-based correlations. The color of each edge denotes statistical significance, evaluated using 999 permutation tests. (G) RF indicates the predictive significance of dominant genus (>5% of total community) as factors influencing L-kynurenine buildup in TBI mice. The relevance of these predictors was determined by the percentage increase in the MSE of variables, with greater MSE% values pointing to more vital predictors. (H) Relative abundance analysis of *Escherichia_Shigella* in gut microbiomes. *P<0.05, **P<0.01, ***P<0.001.

induced intestinal barrier damage. In line with the positive impact on the intestinal barrier, VNS significantly reduced Cldn2 expression but elevated Tjp1 and Ocln expression in colon tissue (Fig. 4F-H). As demonstrated in Fig. 4I-L, TBI markedly decreased the expression of Claudin-5, occludin, and ZO-1 proteins in colonic tissue, suggesting heightened gut permeability, which was mitigated by VNS. Collectively, these data suggest that VNS effectively mitigated TBI-related intestinal barrier dysfunction.

VNS improved cognitive impairment by suppressing gut microbiota dysbiosis after TBI: To establish the causal relationship between VNS improving cognitive impairment after TBI and remodeling gut microbiota, we used an antibiotic mixture (a combination of 5 mg/mL neomycin sulfate, 1.25 μg/mL natamycin, and 5 mg/mL bacitracin) to clear the gut microbiota of TBI mice. The Longa score was conducted at post-injury day 10, with behavioral testing of animals performed from days 14 to 20 following TBI (Fig. 5A). The TBI group had elevated

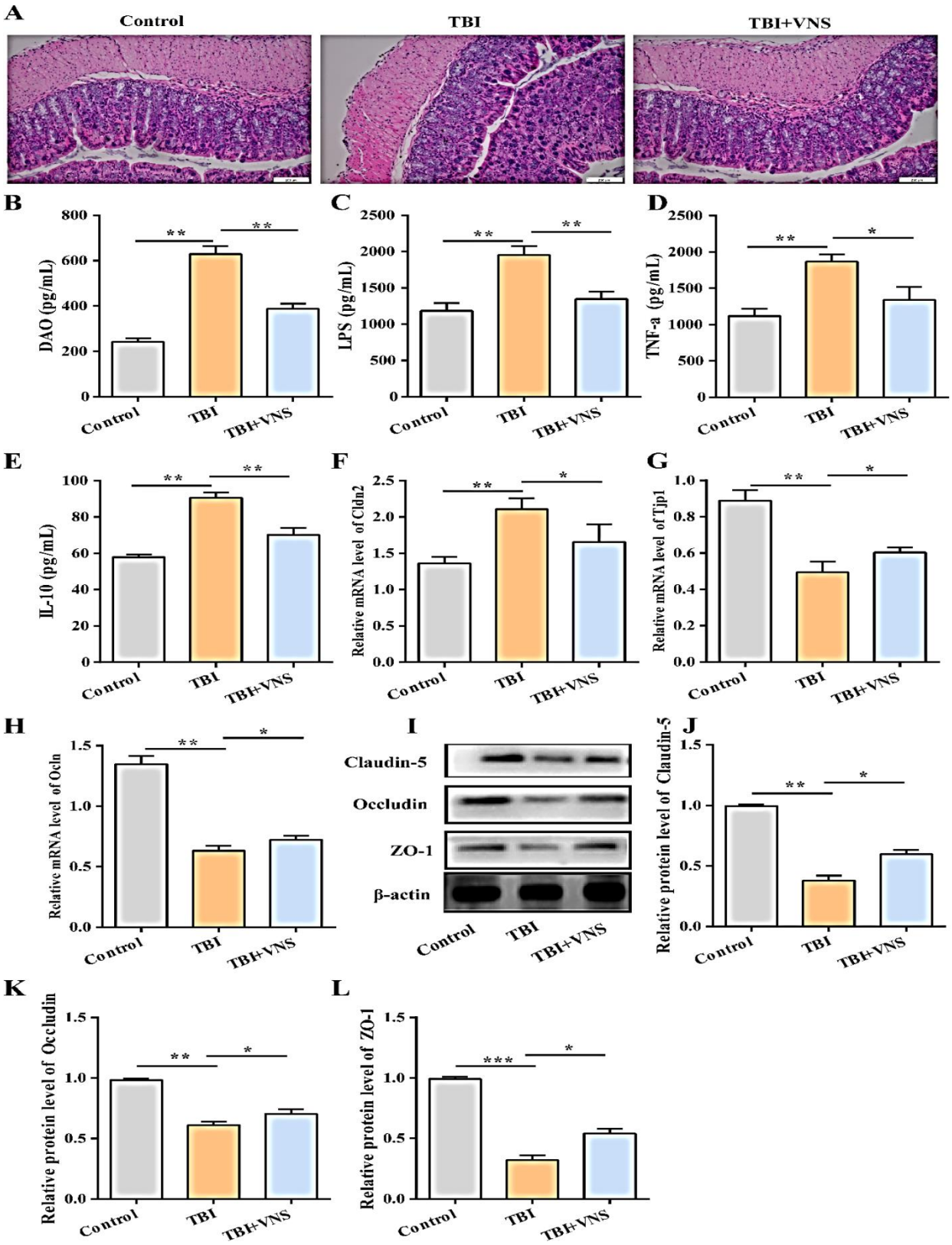

Fig. 4: VNS protected intestinal barrier function in TBI mice. (A) H&E-stained colon tissue sections depicting pathological variations across three groups (n=8/group). Scale bar: 200 μm. (B-E) The serum expression of DAO (B), LPS (C), TNF-α (D), and IL-10 (E) was measured using ELISA. (F-H) Relative mRNA expression levels of Cldn2 (F), Tjp1 (G), and Ocln (H) in colonic tissues was assessed using qRT-PCR. (I) Western blot bands representing Claudin-5, occludin, and ZO-1 in colon tissue. (J-L) Statistical examination of Claudin-5 (J), occludin (K), and ZO-1 (L) protein levels within colon samples. *P<0.05, **P<0.01, ***P<0.001.

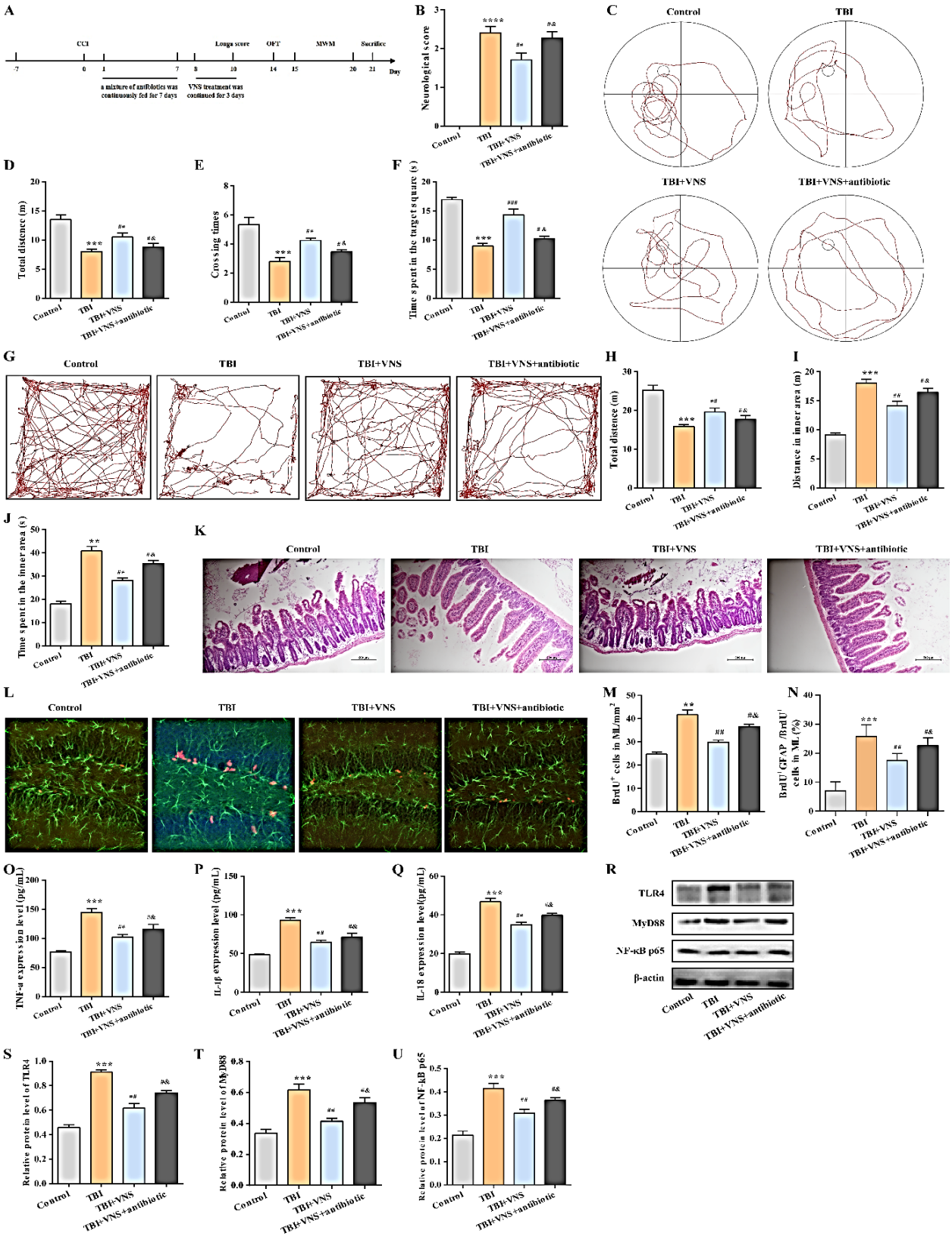

Fig. 5: VNS improved cognitive impairment by suppressing gut microbiota dysbiosis and neuroinflammation after TBI. (A) Timeline of the experimental procedure. (B) Neurological function of four groups was analyzed by Longa neurological score (n=8/group). (C) Representative trace chart of MWM test of the four groups. (D-F) Quantitative analysis of total distance (D), times of crossing the platform (E), and duration in the target quadrant (F) in the MWM test. (G) Trace chart of mice in the OFT experiment. (H-J) Quantitative evaluation of total distance (H), central motion distance (I), and duration within the center (J) during the OFT experiment. (K) Representative H&E staining images of four groups of colon tissues showing pathological changes. Scale bar: 500 μm. (L) Confocal micrographs illustrate dual immunofluorescence for BrdU (red) and GFAP (green) within the dentate gyrus. scale bars: 200 μm. (M) Statistical diagram of $BrdU^+$ cells density in the ML. (N) The ratio of $(BrdU^+GFAP^+)/BrdU^+$ in the ML. (O-Q) The hippocampal expression of TNF-α (O), IL-1β (P), and IL-18 (Q) was measured using ELISA. (R) Western blot bands representing TLR4, MyD88, and NF-κB p65 in the hippocampus. (S-U) Statistical examination of TLR4 (S), MyD88 (T), and NF-κB p65 (U) protein expression in the hippocampus. **P<0.01, ***P<0.001, and ****P<0.0001 vs. Control, #P<0.05, ##P<0.01, and ###P<0.001 vs. TBI, &P<0.05 vs. TBI+VNS.

Longa scores in comparison to controls, and VNS treatment significantly reduced Longa scores. Nevertheless, following antibiotic-mediated depletion of the gut microbiota, the neuroprotective effects elicited by VNS exhibited compromised efficacy in restoring cognitive function parameters (Fig. 5B). The MWM test was performed to assess how antibiotic-induced depletion of gut microbiota affects spatial memory and cognitive function in mice with TBI (Fig. 5C). In the TBI group, the total distance traveled, times of crossing the platform, and duration in the target quadrant were all notably less compared with the control group. VNS notably improved memory deficits induced by TBI. However, antibiotic-induced gut microbiota depletion partially diminished VNS-mediated memory improvement (Fig. 5D-F). Next, the OFT was used to assess cognitive impairment related behavior after antibiotics cleared the gut microbiota (Fig. 5G). The TBI group's total travel distance was notably less than that of the control group, with extended central motion distance and duration within the center. Administration of VNS significantly improved these conditions. However, the use of antibiotics significantly suppressed the positive effects of VNS (Fig. 5H-J).

Furthermore, H&E staining was conducted to assess pathological modifications in colonic tissue following the removal of gut microbiota by antibiotics. The colon tissue structure in the control group was clear, with no significant changes in morphology. The colon mucosa in the TBI group was incomplete, with some epithelial cell shedding, and a significant infiltration by inflammatory cells. These morphological changes were diminished in TBI mice as a result of VNS treatment. Noticeably, the use of antibiotics significantly inhibited the reversal effect of VNS (Fig. 5K). We further assessed the proliferation of astrocytes using double immunofluorescence staining for BrdU (red) and GFAP (green) (Fig. 5L). The number of $BrdU^+$ cells and the percentage of $BrdU^+GFAP^+$ co-labelled cells to $BrdU^+$ cells were significantly increased in ML of the TBI group compared to the control group, while VNS significantly inhibited astrocyte proliferation. However, the use of antibiotics significantly weakened the inhibitory effect of VNS on astrocyte proliferation (Fig. 5M-N). Further analysis revealed a significant elevation of TNF-α, IL-1β, and IL-18 in the TBI mice relative to the control mice. In the meantime, VNS significantly diminished proinflammatory cytokine levels, yet antibiotics prevented the effect (Fig. 5O-Q). In TBI mice, the protein levels of TLR4, MyD88, and NF-κB p65 in the hippocampus were notably elevated. This process was nonetheless suppressed by VNS. Surprisingly, this effect was somewhat reversed upon treatment with the antibiotic mixture (Fig. 5R-U). In conclusion, these findings indicate that VNS ameliorated cognitive dysfunction through modulation of gut microbial dysregulation after TBI.

DISCUSSION

VNS is a technique for neuro-modulation that has been extensively applied in treating refractory epilepsy, depression, and various other neuropsychiatric conditions (Castillo *et al.*, 2022). Moreover, emerging evidence indicates that VNS demonstrates therapeutic benefits for cognitive deficits in animals with TBI (Korupolu *et al.*, 2024). However, it remains unclear how VNS impacts cognitive dysfunction after TBI. Our study provides the first experimental demonstration that VNS can mitigate post-TBI cognitive deficits, possibly by remodeling gut microbiome, enhancing intestinal immune defenses, and inhibiting neuroinflammatory responses.

Neuroinflammation is a key contributor to the pathological mechanisms underlying secondary injury following TBI (Wu *et al.*, 2025). Herein, we noted a notable reduction in neuron numbers within the hippocampal CA1 and CA3 of TBI mice, potentially attributable to neuroinflammatory responses in the hippocampus. VNS increased the number of neurons, thereby improving cognitive impairments induced by TBI. The expression of c-Fos can be rapidly activated during neuroinflammation, especially after stimulation by inflammatory factors such as IL-1β (Jagot *et al.*, 2023). In our study, $c\text{-}Fos^+$ cell expression was increased in the hippocampal granulosa cell layer of TBI mice. Astrocytes, as the homeostatic cells of the central nervous system, rapidly proliferate and activate during the early stages of TBI (Xie *et al.*, 2022). The data revealed a marked elevation in $BrdU^+GFAP^+$ cell ratios within the molecular layer of the dentate gyrus in TBI mice, suggesting that TBI induced astrocyte proliferation. Quantitative analyses revealed significant upregulation of TNF-α, IL-1β, and IL-18 expression in post-TBI hippocampal specimens. TLR4, widely present in the brain, triggers inflammation and plays a key role in neuroinflammation during central nervous system disorders and injuries in both humans and animals (DeClue *et al.*, 2020). The findings of Jiang et al. showed that TLR4, MyD88, and nuclear NF-κB p65 were upregulated in the hippocampus of TBI rats, which is consistent with our results (Jiang *et al.*, 2022). VNS significantly reduced the expression of $c\text{-}Fos^+$ cells, inhibited astrocyte proliferation, reduced pro-inflammatory cytokine release, and suppressed the TLR4/MyD88/NF-κB pathway activation. These results indicate that VNS can inhibit the TBI-induced hippocampal neuroinflammation.

Gut microbes crucially influence neuroimmune function through gut-brain pathways. Dysbiosis is implicated in neuropsychiatric disease development (Stavroulaki *et al.*, 2023). TBI diminishes gut microbiota diversity in humans and animals (Lin *et al.*, 2025). VNS impacts the brain directly and opens up new treatment options by modulating the gut-brain axis and gut microbiota interaction (Wang *et al.*, 2024). Our findings update the knowledge that VNS improves cognitive impairment after TBI by regulating gut microbiota. According to the β-diversity analysis, TBI formed a unique microbial community in mice, indicating its possible impact on the diversity of gut microbiota. VNS therapy partially improved gut microbiota imbalance caused by TBI, suggesting a positive impact of VNS on TBI-related gut dysbiosis. Consistent with the observations of Houlden et al. (Houlden *et al.*, 2016), we found changes in the composition of *Firmicutes* and P*roteobacteria* in TBI mice, particularly marked by a surge in *Enterobacteriaceae* populations. Furthermore, an increase in *Escherichia_Shigella* was connected to impairments in cognition and learning and memory ability. VNS significantly reduced the abundance of *Escherichia-Shigella* in TBI mice, thus revealing a novel therapeutic

target for TBI interventions. Therefore, we believe that VNS may alleviate cognitive impairment after TBI by remodeling the gut microbiota.

TBI disrupts gut microbiota, leading to colonic inflammation and increased intestinal permeability, weakening the mucosal barrier (Du *et al.*, 2023). Similarly, in our results, TBI mice exhibited significant colonic pathological changes and intestinal inflammation, with higher serum activities of DAO, LPS, TNF-α, and IL-10. Cldn2, Tjp1, and Ocln are key components of intestinal tight junctions, regulating intestinal permeability and barrier function (Kramberger *et al.*, 2024). In our study, the intestinal permeability was increased in TBI mice, characterized by a significant rise in Cldn2 levels and a marked decrease in Tjp1 and Ocln expression. Tight junctions (TJs), composed of proteins such as occludin, ZO-1, and claudin, are vital for intestinal epithelial cell adherence (Ahmadifar *et al.*, 2023). In TBI mice, colon tissue showed marked reduction of Claudin-5, Occludin, and ZO-1 proteins, reflecting TBI-induced intestinal barrier damage. Consistent with its gut microbiota remodeling effects, VNS alleviated colonic histopathological changes, suppressed intestinal inflammation, improved epithelial barrier function, and restored barrier integrity.

Studies have shown that remodeling gut microbiota through probiotics or gut microbiota transplantation (FMT) may improve health in both humans and animals (Chaitman and Gaschen, 2021). Our previous research found that VNS improved the TBI-caused gut dysbiosis. To further clarify the relationship between VNS improving cognitive impairment after TBI and remodeling the gut microbiota structure, we conducted a microbe-clearing experiment. We found that after using antibiotics to clear gut microbiota, the effects of VNS in improving neurological deficits and cognitive impairments, reducing pathological changes in colonic tissue, and inhibiting neuroinflammation were significantly weakened. These results suggest that VNS may improve cognitive impairment after TBI by remodeling the gut microbiome. This establishes a causal necessity of gut microbiota in VNS efficacy.

Conclusions: In summary, our research indicates that VNS can improve cognitive impairment after TBI through the "microbiome-gut immune barrier-neuroinflammation" axis, supporting the use of VNS as an intervention to manage secondary injuries post-TBI in both humans and companion animals.

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