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# Median nerve electrical stimulation improves traumatic brain injury by reducing TACR1 to inhibit nuclear factor-κB and CCL7 activation in microglia

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**Summary.** The existing report elucidates that median nerve electrical stimulation (MNS) plays a role in treating traumatic brain injury (TBI). Herein, we explored the mechanism of MNS in TBI. A TBI-induced coma model (skull was hit by a cylindrical impact hammer) was established in adult Sprague-Dawley rats. Microglia were isolated from newborn Sprague-Dawley rats and was injured by lipopolysaccharide (LPS; 10 ng/mL). Consciousness was assessed by sensory and motor functions. Brain tissue morphology was detected using hematoxylin-eosin staining assay. Ionized calcium binding adapter molecule 1, NeuN and tachykinin receptor 1 (TACR1) level were detected by immunohistochemical assay. Levels of pro-inflammatory and anti-inflammatory factors were measured by enzyme linked immune sorbent assay (ELISA). Levels of TACR1, C-C motif chemokine ligand 7 (CCL7), phosphorylation (p)-P65 and P65 were assessed by quantitative real time polymerase chain reaction (qRT-PCR) and western blot. M1 markers (inducible nitric oxide synthase and CD86) and M2 markers (arginase-1 (Arg1) and chitinase 3-like 3 (YM1)) of microglia as well as the transfection efficiency of short hairpin TACR1 (shTACR1) were assessed by qRT-PCR. Immunofluorescence and flow cytometry assay were used to detect microglia morphology and neuron apoptosis. MNS reduced neuron injury and microglia activation in the TBI-induced rat coma model. MNS reversed the effects of TBI on levels of inflammation-related factors, M1/M2 microglia markers, TACR1, p-P65/P65 and CCL7 in rats. shTACR1 reversed the effects of LPS on inflammation-related factors, M1/M2 microglia markers, microglia activation, neuron apoptosis, p-P65/P65 value and CCL7 level. Our results revealed that MNS improved TBI by reducing TACR1 to inhibit nuclear factor-κB (NF-κB) and CCL7 activation in microglia.

**Key words:** Traumatic brain injury, Median nerve electrical stimulation, Tachykinin receptor 1, Microglia, C-C motif chemokine ligand 7

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## Introduction

Traumatic brain injury (TBI), a kind of severe brain disease, is an important reason for disability and death in the world (Capizzi et al., 2020). In most cases, TBI can lead to temporary or permanent brain impairment, which is disastrous for patients (Capizzi et al., 2020; Scarboro and McQuillan, 2021). Many researches have reported that TBI can result in consciousness loss, coma, sensory and motor dysfunction, which seriously affects patient's life (Khellaf et al., 2019; Robinson, 2021; Scarboro and McQuillan, 2021). Hence, rehabilitation treatment of TBI patients has become one of the key research projects in the medical field. In recent years, many therapies have emerged to treat TBI, chiefly including music therapy (Siponkoski et al., 2020), temperature management (Kochanek et al., 2019) and deep brain stimulation (Kundu et al., 2018). Particularly, neurorehabilitation is the best treatment option for the improvement of motor functions in TBI patients (Hofer and Schwab, 2019). Therefore, the effect of nerve regulation technology on TBI is further explored in this study.

Among many nerve regulation technologies, median nerve electrical stimulation (MNS) is the most widely used to treat TBI in the clinic, which is safe, non-invasive, with strong operability and low cost (Jia et al., 2022; Zhang et al., 2022a). The Median nerve, the only nerve that passes through the carpal tunnel, is the peripheral portal of the central nervous system

(Draghi et al., 2022). Existing evidence indicates that MNS technology has achieved remarkable effectiveness in the clinical treatment of consciousness disorder (Wu et al., 2017; Feller et al., 2021). What's more, MNS technology can regulate brain plasticity by promoting neuron regeneration in TBI disease (Kuo et al., 2021; Jia et al., 2022). Nevertheless, there is still little research to amply illustrate the therapeutic molecule mechanism of MNS in TBI.

A crucial study confirms that microglia, the main immune cells in the central nervous system, are quickly enriched in TBI site when brain injury occurrs, which then leads to neuroinflammation and neuronal dysfunction (Witcher et al., 2021). Reportedly, activated microglia can be classified into two types, including M1 microglia and M2 microglia (Tang and Le, 2016). M1 microglia represent pro-inflammatory and neurotoxic activities, which chiefly manifests as high levels of inducible nitric oxide synthase (iNOS) and CD86 (Shao et al., 2022). By contrast, M2 microglia exert their role in inflammatory suppression, debris elimination and tissue reconstitution, which mainly manifests as high levels of arginase-1 (Arg1) and chitinase 3-like 3 (YM1) (Colonna and Butovsky, 2017; Shao et al., 2022). Hence, preventing microglia from over-activating and promoting the activation of M2 type microglia is one of the important research directions in the treatment of TBI. Presently, a report has supported that electrical stimulation can induce the production of M2 microglia (anti-inflammatory phenotype) in a TBI rat model (Park et al., 2021). Therefore, we speculated that MNS could promote the formation of M2 microglia after the aggregation of microglia in the TBI location. Then, we aimed to find a gene that mediated the activation of microglia.

In our earlier study, the differential expression gene data set in M2 microglia (GSE199400) was analyzed. We found that the expression of C-C motif chemokine ligand 7 (CCL7) was abnormally expressed in M2 microglia. CCL7 can recruit inflammatory cells (for example, monocytes) to strengthen the inflammatory reaction (Liu et al., 2018). Notably, the inhibition of microglia activation is mediated by decreasing CCL7 (Li et al., 2017). However, the connection of CCL7 with MNS-regulated microglia activation is still unclear. Remarkably, previous study delineates that the therapy of electrical stimulation can affect tachykinin receptor 1 (TACR1) expression (Broiz et al., 2012). The inhibition of TACR1 can lessen microglia activation (Zhu et al., 2014). Moreover, TACR1 antagonist synergistically acts with therapeutic electrical stimulation after brain injury (for instance, TBI) (Klyukin et al., 2019). A finding shows that electrical stimulation can reduce TACR1 level (Suzuki, 2002). Next, we focused on whether TACR1 could regulate the abnormal expression of CCL7. An earlier report reveals that TACR1 can stimulate cell pro-inflammatory signal through activating nuclear factor-κB (NF-κB) (Williams et al., 2007). More interestingly, the transcriptional activity of NF-κB is an important way to regulate CCL7 (Li et al., 2018).

Collectively, the purpose of this research aimed to explore whether MNS improved TBI by reducing TACR1 to inhibit NF-κB and CCL7 activation in microglia.

## Materials and methods

### *Ethic statement*

Adult and newborn Sprague-Dawley rats were obtained from Hangzhou Medical College (Zhejiang, China). Rats were fed at a 12h light-dark cycle room (25°C room temperature and 47% humidity). Rats could freely gain standard water and food. All experimental operations on rats were performed in accordance with the China Council on Animal Care and Use guidelines and were approved by the Ethics Committee of Zhejiang Baiyue Biotech Co., Ltd for Experimental Animals Welfare (ZJBYLA-IACUC-20220815).

### *Establishment of TBI-induced coma model and MNS treatment*

After feeding 1 week, 160 adult rats (7 weeks old, 250-300 g, male) were randomly divided into four groups (40 rats per group), including the sham group, the model group, the model + sham group and the model + MNS group. All rats were anesthetized by intraperitoneally injecting with pentobarbital sodium (50 mg/kg body weight; P3761; Sigma-Aldrich, MO, USA) (Ge et al., 2021). The sham group: rats were performed a sham operation, which was the same surgical procedure with the model group but without hitting the skull. The model group: rats were applied to establish TBI-induced coma model. According to an early study with minor modifications, the skull of rat was exposed (5 mm vertical incision). Then, a syringe needle was employed to mark the crossover hit point (2 mm adjacent to the left midline and 1 mm anterior to the coronal suture). After that, the marked point in plastic spacer was hit by a cylindrical impact hammer (400 g; dropping at a 40-44 cm height) along a vertical metal bar, which then led to a concave fracture of the skull. Finally, the incision was closed (Feng et al., 2015). The model + sham group: rats were performed the same surgical procedure with the model group but without MNS treatment (only needling and no electrical current output). The model + MNS group: after TBI 30 min, rats were performed unilateral MNS treatment for 6 h (Feng et al., 2015). MNS treatment was performed by a low frequency electrical stimulator (ES-420; ITO Physiotherapy and Rehabilitation, Tokyo, Japan). Briefly, the midpoint of the volar wrist crease was connected to the stimulator by an acupuncture needle. The parameters were set as: frequency, 30 Hz; pulse width, 0.5 ms; electrical current, 1.0 mA; total time, 15 min.

### *Consciousness evaluation*

After TBI 30 min and MNS treatment 1h,

consciousness of rat was assessed in accordance with early researches (Feng et al., 2015; Feng and Du, 2016). According to sensory and motor functions, consciousness was classified into I-VI grades. I: Rats could freely engage in daily activity. II: Activity was reduced. III: Reduced activity and motor incoordination. IV: Righting reflex could be induced; rats could stand up. V: Righting reflex vanished; rats could react to noxious stimuli. VI: No reaction to noxious stimuli. Finally, rats in grade V and VI (lasting at least 30 min) were regarded as a coma state, which were used for further experiments. After the consciousness evaluation of MNS treatment, rats were sacrificed using cervical dislocation to collect the brain tissues.

### *Hematoxylin-eosin (HE) staining assay*

The morphology of brain tissue in rat was detected using HE staining assay kit (C0105S; Beyotime, Shanghai, China). Harvested brain tissues were embedded in paraffin (A601888; Sangon Biotech, Shanghai, China) and then were sliced by a tissue slicer (5-10 μm thickness; HS-S7220-B; Shenyang Hengsong Technology, Liaoning, China). According to manufacturer's instructions, tissue slices were removed from wax by xylene (A530011; Sangon Biotech, Shanghai, China) for 10 min (two times). Afterward, samples were orderly immersed with different concentrations of ethanol and distilled water. Next, samples were orderly incubated with hematoxylin staining solution (10 min) and eosin staining solution (2 min). After dehydrating, xylene was used to treat samples for 5 min. Neutral gum (E675007; Sangon Biotech, Shanghai, China) was employed to seal samples. Finally, images were photographed by a light microscope (magnification, ×100; LV150; Nikon Inc., Tokyo, Japan).

### *Immunohistochemical assay*

Immunohistochemical assay was applied to detect the expressions of ionized calcium binding adapter molecule 1 (Iba1), NeuN and TACR1 in brain tissues of rats. Briefly, tissue slices were dewaxed to water. Then, samples were orderly treated with 0.3% $H_2O_2$ (for 10 min at 25°C; ST858-1L; Beyotime, Shanghai, China) and 0.01 mol/L sodium citrate buffer (for 15 min at 95°C; C1010; Solarbio, Beijing, China). After immersing by 1 × phosphate buffer saline (PBS; P1020; Solarbio, Beijing, China) for 5 min, samples were incubated with 5% bovine serum albumin (BSA) blocker (37525; Thermo Fisher Scientific, MA, USA) for 1h at 25°C. Afterward, antibodies of Iba1 (ab178846; 1:2000; abcam, Cambridge, UK), NeuN (ab177487; 1:3000; abcam, Cambridge, UK) and TACR1 (PA1-16715; 1:500; Thermo Fisher Scientific, MA, USA) were applied to incubate samples overnight at 4°C. Samples were incubated with secondary antibody (ab205718; goat anti rabbit; 1:10000; abcam, Cambridge, UK) for 30 min at 37°C. After that, samples were orderly treated with streptavidin-biotin complex-horseradish peroxidase reagent (for 30 min at 37°C; P0615; Beyotime, Shanghai, China) and 3,3N-diaminobenzidine tertrahydrochloride staining solution (avoiding light for 15 min at 25°C; P0202; Beyotime, Shanghai, China). In the next step, samples were stained by hematoxylin staining solution for 5 min. After dehydrating, samples were rendered transparent with xylene and then sealed by neutral gum. Finally, images were photographed by a light microscope (magnification, ×100).

### *Isolation and culture of rat microglia*

According to previous research, microglia were isolated from rat mixed glial cultures by the shaking method (Brás et al., 2020). Briefly, newborn Sprague-Dawley rats (two days old, male) were decapitated. The brain tissues were separated from the skull, then treated with 0.25% trypsin (C0203; Beyotime, Shanghai, China) for 15 min at 37°C. After that, harvested rat brain-derived mixed glial cells were seeded into T75 flasks, which were cultured by dulbecco's modified eagle medium (DMEM; SH30022.01B; Hyclone, UT, USA) with 10% fetal bovine serum (FBS; 10100147; Thermo Fisher Scientific, MA, USA) for 21 days. In the next step, microglia were isolated from rat mixed glial cultures. Flasks were shaken on a shaker (TS-1; Haimen Kylin-Bell Lab Instruments, Jiangsu, China) at 150 rpm for 2h (37°C). Afterward, microglia were obtained after culturing for 14 days. Isolated microglia were cultured by DMEM/F12 medium (PM150310B; Procell Life Science and Technology, Hubei, China) with 10% FBS, maintaining at 37°C with 5% $CO_2$ atmosphere.

### *Cell treatments*

Following incubation, microglia were divided into four groups. The control group: microglia were normally cultured. The lipopolysaccharide (LPS) group: microglia were incubated with LPS (10 ng/mL; 916374; Merck, Shanghai, China) for 4h (Jiang et al., 2021). The LPS + short hairpin negative control (shNC) and LPS + shTACR1 group: microglia were incubated with LPS for 4h and then transfected with shNC or shTACR1 for 24h. ShTACR1 was bought from Santa Cruz Biotechnology (including shTACR1-1, shTACR1-2 and shTACR1-3; sc-36069-SH; Shanghai, China) and the shNC (as control) was complimentary from Santa Cruz Biotechnology. On the other hand, according to above groups, after transfection, microglia was co-cultured with rat neurons for 24h in 24-well transwell chambers (CLS3396; 0.4 μm pore; Merck, Shanghai, China). Microglia was cultured on the upper chamber. Neurons were cultured on the lower chamber. Rat neurons were obtained from Procell Life Science and Technology (CP-R105; Hubei, China), which were cultured by neurons special medium (CM-R105; Procell Life Science and Technology, Hubei, China) at 37°C with 5% $CO_2$

atmosphere.

### *Cell transfection*

Microglia ($5\times10^5$ cells/well) were inoculated into 6-well plates for 24h. According to the above groups, microglia were obtained for the corresponding treatments. Lipofectamin 3000 transfection reagent (L3000001) and reduced serum medium (11058021) were bought from Thermo Fisher Scientific (MA, USA), which were used for transfection. Briefly, solution A (5 μg shNC/shTACR1 and 125 μL reduced serum medium) and solution B (10 μL Lipofectamin 3000 reagent and 125 μL reduced serum medium) were prepared in advance. After that, solution A and solution B were fully mixed and then placed for 15 min. Finally, the complexes of solution A and solution B were added into the microglia, culturing for 24h at 37°C with 5% $CO_2$ atmosphere.

### *Enzyme linked immune sorbent assay (ELISA)*

The levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, IL-4, IL-10 and transforming growth factor (TGF)-β in brain tissues (injury site) and microglia were measured by ELISA. Rat IL-1β (EK301B), IL-6 (EK306), TNF-α (EK382), IL-4 (EK304), IL-10 (EK310) and TGF-β (EK981) ELISA kit were bought from Lianke Biology (Zhejiang, China). According to the manufacturer's instructions, samples and reagents were balanced to room temperature before testing. Briefly, 2-fold diluted standard (100 μL), standard diluent (100 μL) and sample (100 μL) were added into standard, blank and sample wells. Incubated samples were shaken on a shaker at 200 rpm for 2h. Then, chromogenic substrate (100 μL each well) was added into samples, avoiding light and incubating for 20 min. Terminating liquid (100 μL each well) was added into samples. Within 30 min, absorbance (OD; 450 nm and 570 nm) was measured by a microplate reader (HTS-XT; Bruker Optics, Rheinstetten, Germany). The calibrated OD value was $OD_{450}$-$OD_{570}$.

### *Quantitative real time polymerase chain reaction (qRT-PCR)*

Total RNA extractor kit (B511311; Sangon Biotech, Shanghai, China) was applied to extract RNA. Evolution 260 Bio Spectrophotometer (840-211000; Thermo Fisher Scientific, MA, USA) was applied to detect RNA concentration. RNA was mixed with one step qRT-PCR probe reagents (B639278; Sangon Biotech, Shanghai, China), which was detected by the qRT-PCR instrument (MJOpticon2, Bio-Rad Inc., CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was used for normalization. Data were analyzed by the formula: $2^{-\Delta\Delta Ct}$ (Feng et al., 2015). Primers (Table 1) were synthesized by Thermo Fisher Scientific (MA, USA).

### *Western blot analysis*

The brain tissues of rats were ground to extract total

**Table 1.** The primer sequences of related genes.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| CD86 | AAGACATGTGTAACCTGCACCA | GCCCAGGTACTTGGCATTCA |
| iNOS | ATGCAGAATGAGTACCGGGC | GCGTTTCGGGATCTGAATGC |
| Arg1 | AGAGGCTCGCAGGGAAGA | GCTGTCATTGGGGACATCCA |
| YM1 | ATGGCCTCAACTTGGACTGG | TCACGAGCTGCCCCATTATC |
| TACR1 | ACTCCTCTGACCGCTACCAT | CAGACGGAACCTGTCGTTGA |
| GAPDH | CCGCATCTTCTTGTGCAGTG | ACCAGCTTCCCATTCTCAGC |

Arg1, arginase-1; iNOS, inducible nitric oxide synthase; YM1, chitinase 3-like 3; TACR1, tachykinin receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Table 2.** Antibodies used in this study.

| Name | Catalog | Molecular weight | Dilution | Manufacturer |
|---|---|---|---|---|
| *TACR1* | sc-514453 | 46 kDa | 1/1000 | Santa Cruz, China |
| *p-P65* | 3033 | 65 kDa | 1/1000 | CST, USA |
| *P65* | 8242 | 65 kDa | 1/1000 | CST, USA |
| *CCL7* | PA5-86885 | 11 kDa | 1/500 | Invitrogen, USA |
| *GAPDH* | ab8245 | 37 kDa | 1/5000 | abcam, UK |
| goat anti rabbit | ab97051 | — | 1/10000 | abcam, UK |
| goat anti mouse | ab96879 | — | 1/10000 | abcam, UK |

TACR1, tachykinin receptor 1; p-P65, phosphorylation-P65; CCL7, C-C motif chemokine ligand 7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

protein. Microglia ($5\times10^5$ cells/well) were seeded into 6-well plates and then cultured for 24h. According to above groups, microglia were obtained for the corresponding treatments. Briefly, total protein was extracted by protein extraction kit (C006225; Sangon Biotech, Shanghai, China). The concentration of total protein was measured by protein concentration assay kit (P0010; Beyotime, Shanghai, China). After that, total protein was separated by gel reagent (P0012A; Beyotime, Shanghai, China) and was transferred into membranes (0.45 μm; YA1711; Solarbio, Beijing, China). In the next step, membranes were orderly incubated with 5% BSA blocker (for 1h at 25°C), primary antibody (for 2h at 25°C) and secondary antibody (for 1h at 25°C). Related antibodies are displayed in Table 2. The internal reference was GAPDH. Protein blots were detected by electro-chemiluminescence reagent (32209; Thermo Fisher Scientific, MA, USA), which were visualized by a gel imaging analysis system (GeneGenius; Baijing Biotechnology Inc., Beijing, China) and analyzed by ImageJ software (version 1.8.0; National Institutes of Health, MD, USA).

### *Immunofluorescence assay*

Microglia were grown on cover glass. As previously described, microglia were used for the corresponding treatments. After climbing, cells were fixed by 4% paraformaldehyde (P0099; Beyotime, Shanghai, China) for 15 min and were rendered transparent with Triton X-100 (P0096; Beyotime, Shanghai, China) for 10 min. Then, cells were blocked using immunol staining blocking buffer (P0102; Beyotime, Shanghai, China) for 1h. Afterward, cells were orderly incubated with the primary antibodies of Iba1 (1:1000) and F-actin (ab205; 1:1000; abcam, Cambridge, UK) overnight at 4°C and secondary antibodies of goat anti rabbit (avoiding light; ab150077; Alexa Fluor® 488; 1:500; abcam, Cambridge, UK) and goat anti mouse (avoiding light; ab150115; Alexa Fluor® 647; 1:200; abcam, Cambridge, UK) for 2h at 25°C. Next, cells were stained by 4', 6-diamidino-2-phenylindole (DAPI) staining solution (C1006; Beyotime, Shanghai, China) for 5 min at room temperature. Finally, a confocal fluorescent microscope (magnification, ×400; TCS SP8; Leica Camera AG Inc., Solms, Germany) was applied to capture the images.

### *Flow cytometry assay*

Neuron apoptosis was measured by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (40302ES60; Yeasen Biotechnology, Shanghai, China). Neurons were digested by 0.25% trypsin (without ethylene diamine tetraacetic acid; C0205; Beyotime, Shanghai, China). After centrifuging, cells were counted ($5\times10^5$ cells/sample). Cells were suspended by 1 × binding buffer (100 μL). Annexin V-FITC (5 μL) and PI staining solution (10 μL) were added into cells, avoiding light and incubating for 12 min at 25°C. In the next step, 1 × binding buffer (400 μL) was mixed with cells, then the cells were placed on the ice to detected cell apoptosis by a flow cytometer (FACSCanto II; BD Inc., NY, USA).

### *Statistical analysis*

All measurement data were described by mean ± standard deviation and calculated by GraphPad software (version 8.0; Graphpad Software Inc., CA, USA). One-way ANOVA was applied to compare the data between multiple groups. $P<0.05$ was considered statistically significant difference.

## Results

### *MNS reduced neuron injury and microglia activation in TBI-induced rat coma model.*

As shown in Table 3, the effect of MNS on the recovery of consciousness in rats with TBI-induced coma was assessed. Our results determined that the number of revived rats in model group, model + sham group and model + MNS group was 11, 14 and 25. And the number of rats in coma state was lessened under the treatment of MNS (11 rats) in contrast to model + sham group (22 rats). These outcomes indicated that MNS was beneficial to recover consciousness.

According to HE staining results, the morphology of brain tissues in rats was manifested as focal necrosis and inflammatory cell infiltration in the model group compared to the sham group, while these effects were alleviated by MNS (Fig. 1A). What's more, the areas of microglia activation marker (Iba1), neuron marker (NeuN) and TACR1 were assessed by immunohistochemical assay (Fig. 1B-E). Our data revealed that there were increases of Iba1 and TACR1 area as well as a decrease of NeuN area in the model group relative to the sham group (Fig. 1B-E, $P<0.001$), which were overturned by MNS treatment (Fig. 1B-E, $P<0.001$).

### *MNS reversed the effects of TBI on inflammation-related factors, M1/M2 microglia markers, TACR1, phosphorylation (p)-P65/P65 and CCL7 in rats.*

Furthermore, the effect of MNS on inflammation-

**Table 3.** Effect of MNS on the recovery of consciousness in rats with TBI-induced coma.

| Group | Revived (n) | Coma | |
|---|---|---|---|
| | | Level V (n) | Level VI (n) |
| Model | 11 | 13 | 12 |
| Model + sham | 14 | 12 | 10 |
| Model + MNS | 25 | 7 | 4 |

MNS, median nerve electrical stimulation; TBI, traumatic brain injury.

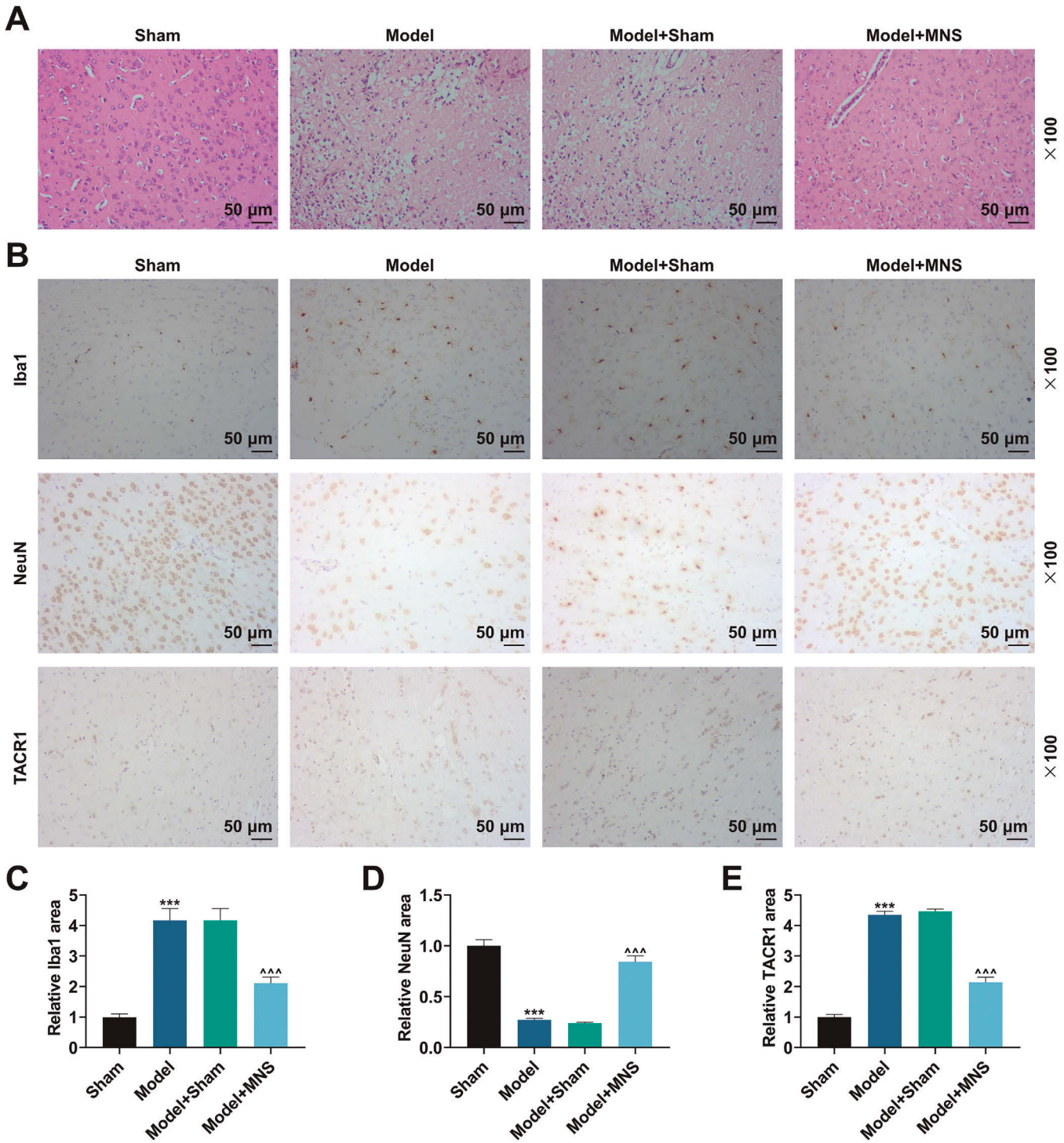


**Fig. 1.** MNS reduced neuron injury and microglia activation in a TBI-induced rat coma model. **A-E.** Rats were divided into four groups, including the sham group: rats were performed the same surgical procedure as the model group but without hitting skull. The model group: after marking, the rat's skull was hit by a cylindrical impact hammer (40-44 cm height) along a vertical metal bar. The model + sham group: rats were performed same surgical procedure as the model group but without MNS treatment (only needling and no electrical current output). The model + MNS group: after TBI 30 min, rats were performed MNS treatment for 6h. **A.** The morphology of brain tissue in rat was detected using HE staining assay. **B-E.** Immunohistochemical assay was applied to detect the expressions of Iba1, NeuN and TACR1 in brain tissues of rats. ***$P$<0.001 vs. Sham group. ^^^$P$<0.001 vs. Model + Sham group. MNS, median nerve electrical stimulation; TBI, traumatic brain injury; HE, hematoxylin-eosin; Iba1, ionized calcium binding adapter molecule 1; TACR1, tachykinin receptor 1.

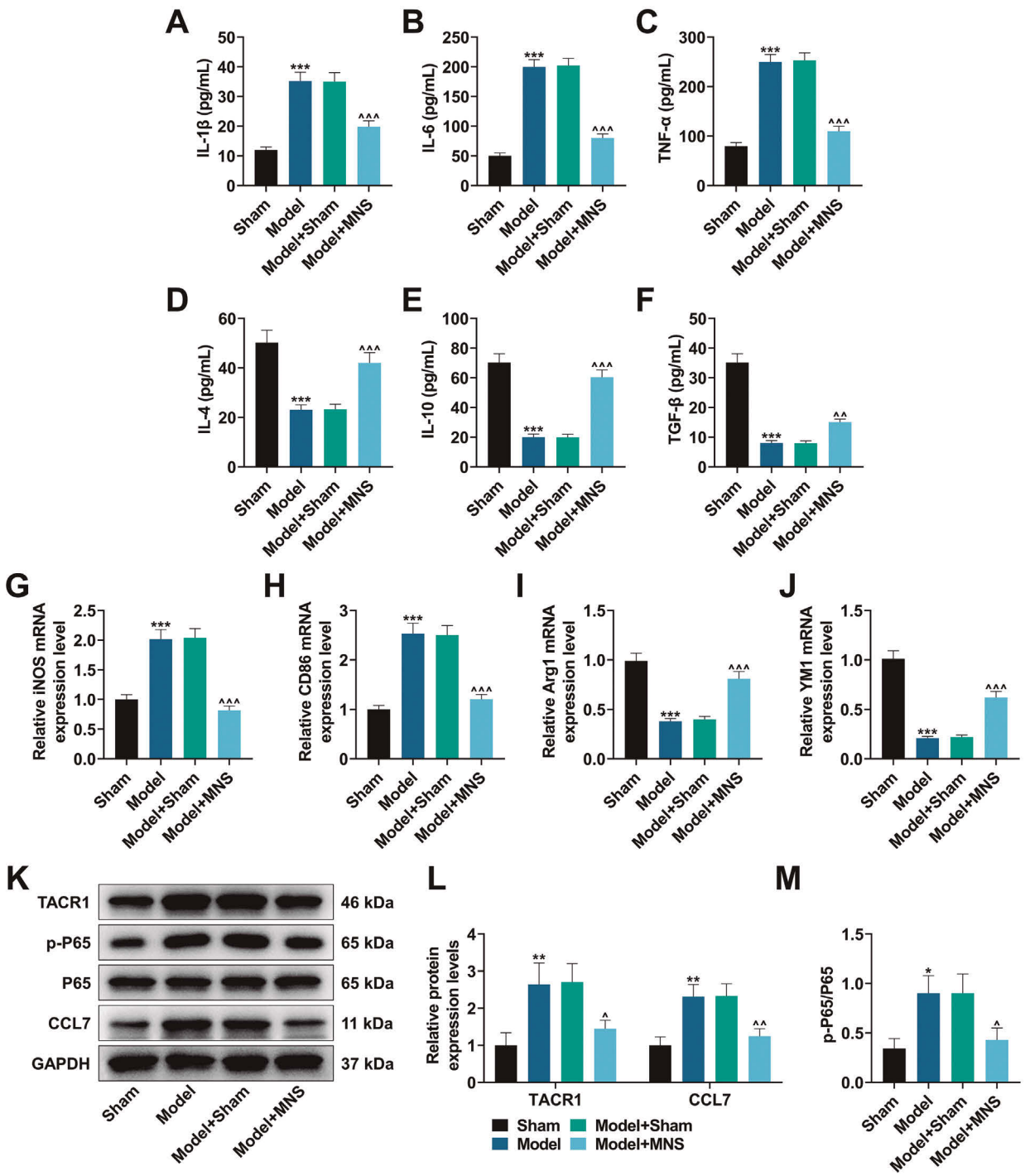


**Fig. 2.** MNS reversed the effects of TBI on inflammation-related factors, M1/M2 microglia markers, TACR1, p-P65/P65 and CCL7 in rats. **A-M.** Rats were divided into four groups, including the sham group: rats were performed the same surgical procedure as the model group but without hitting skull: The model group: after marking, the rat's skull was hit by a cylindrical impact hammer (40-44 cm height) along a vertical metal bar t. The model + sham group: rats were performed the same surgical procedure as the model group but without MNS treatment (only needling and no electrical current output). The model + MNS group: after TBI 30 min, rats were performed MNS treatment for 6h. **A-F.** The levels of IL-1β, IL-6, TNF-α, IL-4, IL-10 and TGF-β in brain tissues (injury site) of rats were assessed by ELISA. **G-J.** The mRNA levels of iNOS, CD86, Arg1 and YM1 in brain tissues of rats were assessed by qRT-PCR. GAPDH was internal reference. **K-M.** Protein levels of TACR1, CCL7, p-P65, P65 and GAPDH were determined by western blot in brain tissues of rats. GAPDH was internal reference. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. Sham group. ^*P*<0.05, ^^*P*<0.01, ^^^*P*<0.001 vs. Model + Sham group. IL, interleukin; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; iNOS, inducible nitric oxide synthase; Arg1, arginase-1; YM1, chitinase 3-like 3; p-P65, phosphorylation-P65; CCL7, C-C motif chemokine ligand 7; ELISA, enzyme linked immune sorbent assay; qRT-PCR, quantitative real time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

related factors was measured in brain injury tissues of rats by ELISA. It was displayed that TBI obviously promoted the pro-inflammatory factor levels of IL-1β, IL-6 and TNF-α (Fig. 2A-C, $P<0.001$) but suppressed the anti-inflammatory factor levels of IL-4, IL-10 and TGF-β (Fig. 2D-F, $P<0.001$) compared with the sham group, whilst these influences were offset by MNS (Fig. 2A-F, $P<0.01$). In addition, according to qRT-PCR results, microglia M1 markers (iNOS and CD86) were elevated (Fig. 2G-H, $P<0.001$) and M2 markers (Arg1

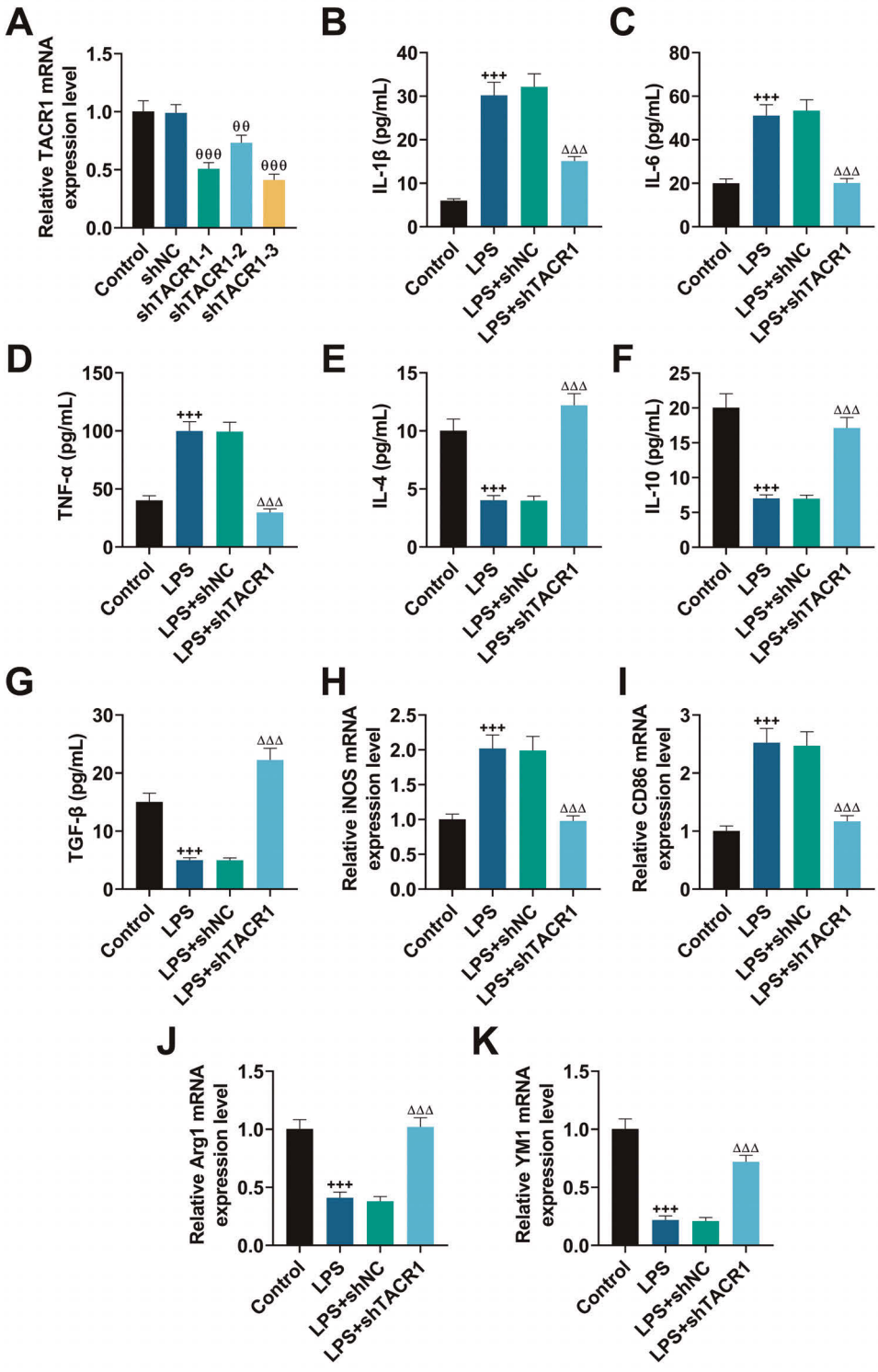


**Fig. 3.** shTACR1 reversed the effects of LPS on inflammation-related factors, M1 markers and M2 markers in microglia. **A.** Microglia were transfected with or without shNC/shTACR1-1/shTACR1-2/shTACR1-3 for 24 h. **A.** The mRNA level of TACR1 in microglia was assessed by qRT-PCR. GAPDH was internal reference. **B-K.** Microglia were divided into four groups. The control group: microglia were normally cultured. The LPS group: microglia were incubated with LPS (10 ng/mL) for 4h. Based on the LPS group, microglia in LPS + shNC and LPS + shTACR1 group were transfected with shNC or shTACR1-3 for 24 h. **B-G.** The levels of IL-1β, IL-6, TNF-α, IL-4, IL-10 and TGF-β in microglia were assessed by ELISA. **H-K.** The mRNA levels of iNOS, CD86, Arg1 and YM1 in microglia were assessed by qRT-PCR. GAPDH was internal reference. $^{\theta\theta}P<0.01$, $^{\theta\theta\theta}P<0.001$ vs. shNC group. $^{+++}P<0.001$ vs. Control group. $^{\Delta\Delta\Delta}P<0.001$ vs. LPS + shNC group. shNC, short hairpin negative control; LPS, lipopolysaccharide.

and YM1) were lessened (Fig. 2I-J, $P<0.001$) more in the model group than in the sham group, yet these trends were counteracted by MNS (Fig. 2G-J, $P<0.001$).

Simultaneously, TACR1, p-P65/P65 and CCL7 levels in brain tissues of rats were assessed by western blot. There were up-regulated effects of TBI on TACR1, p-P65/P65 and CCL7 level relative to the sham group (Fig. 2K-M, $P<0.05$). However, MNS reversed the positive-going influences of TBI on these indexes (Fig. 2K-M, $P<0.05$).

*shTACR1 reversed the effects of LPS on inflammation-related factors, M1/M2 microglia markers and microglia activation.*

In order to explore the effect of TACR1 on LPS-exposed microglia, TACR1 was silenced in microglia.

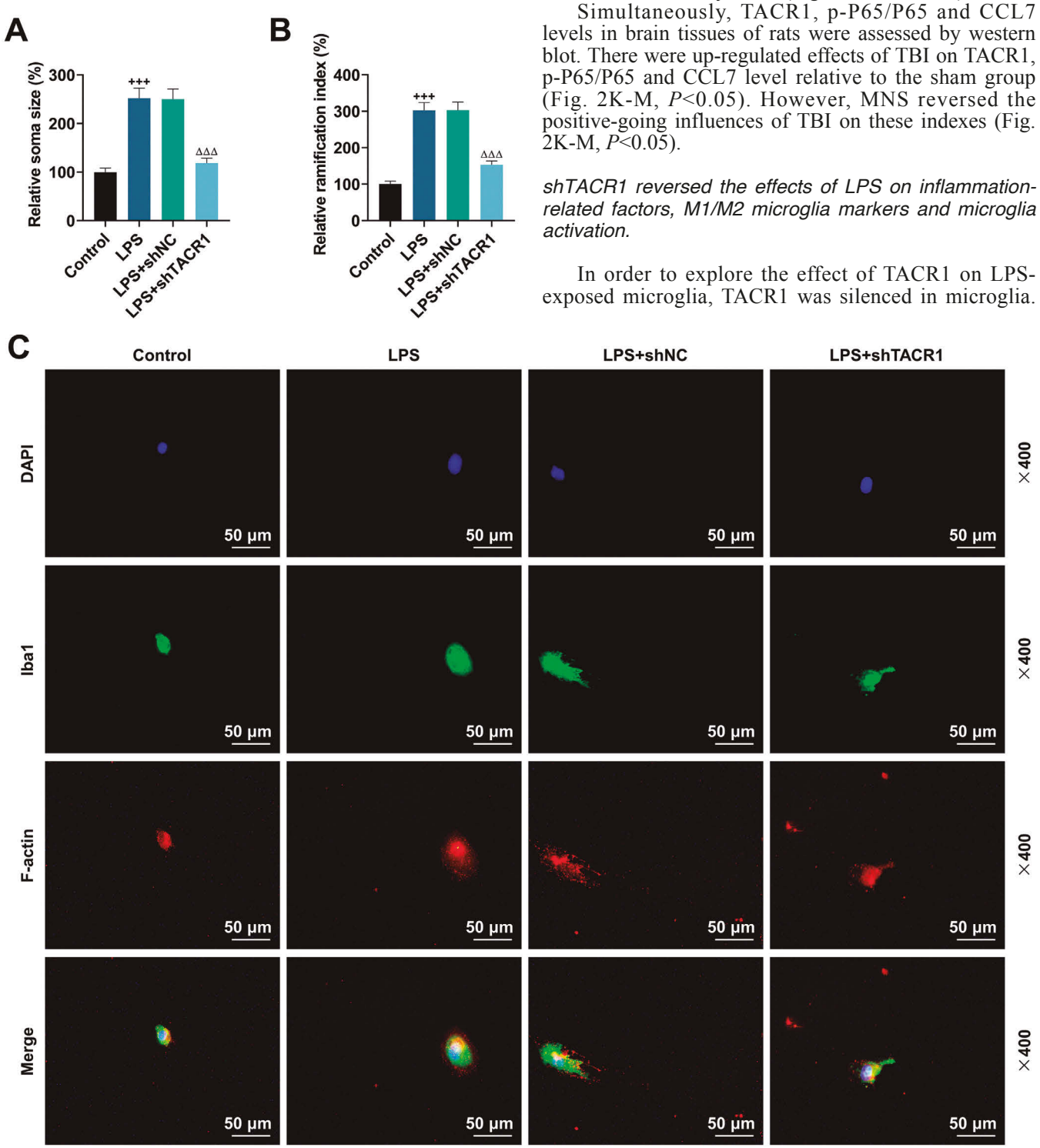


**Fig. 4.** shTACR1 reversed the effect of LPS on microglia activation. **A-C.** Microglia were divided into four groups. The control group: microglia were normally cultured. The LPS group: microglia were incubated with LPS (10 ng/mL) for 4h. Based on the LPS group, microglia in LPS + shNC and LPS + shTACR1 group were transfected with shNC or shTACR1-3 for 24h. **A-C.** Immunofluorescence assay was used to detect microglia morphology. Resolution: 50 $\mu$m, ×400. $^{+++}P<0.001$ vs. Control group. $^{\Delta\Delta\Delta}P<0.001$ vs. LPS + shNC group.

According to qRT-PCR results, TACR1 was successfully silenced in microglia compared with the shNC group (Fig. 3A, $P<0.01$). shTACR1-3 was used for further experiments (the silencing effect was most obvious).

Similarly, inflammatory factors in microglia were detected by ELISA. Our outcomes illustrated that LPS sharply promoted IL-1β, IL-6 and TNF-α level (Fig. 3B-D, $P<0.001$) and decreased IL-4, IL-10 and TGF-β level (Fig. 3E-3G, $P<0.001$) relative to the normal microglia, yet these effects were offset by shTACR1 (Fig. 3B-G, $P<0.001$). On the other hand, there were increased trends of iNOS and CD86 (Fig. 3H-I, $P<0.001$) as well as decreased trends of Arg1 and YM1 (Fig. 3J-K, $P<0.001$) in the LPS-exposed microglia compared to the normal microglia, which were partly restored by shTACR1 (Fig. 3H-K, $P<0.001$).

Additionally, microglia morphology was detected by immunofluorescence assay. It was displayed that soma size and ramification index were increased more in the LPS group than that in the control group (Fig. 4A-C, $P<0.001$). Nevertheless, in LPS-exposed microglia, there were decreases of soma size and ramification index under the action of shTACR1 relative to the LPS + shNC group (Fig. 4A-C, $P<0.001$). It could be seen that LPS could promote microglia activation, which was offset by shTACR1.

### *shTACR1 reversed the effects of LPS on neuron apoptosis as well as levels of p-P65/P65 and CCL7 in microglia.*

Meanwhile, the effect of LPS-exposed microglia on neuron apoptosis was measured by flow cytometry assay. Our results demonstrated that there was up-regulation of neuron apoptosis in the LPS group relative to the control group (Fig. 5A,B, $P<0.001$), while this influence was overturned by shTACR1 (Fig. 5A,B, $P<0.001$). On the other hand, western blot results represented that the levels of p-P65/P65 and CCL7 were elevated more in LPS-exposed microglia than in normal microglia (Fig. 5C-E, $P<0.05$), yet these effects were restored by shTACR1 (Fig. 5C-E, $P<0.05$).

## Discussion

TBI refers to brain injury caused by external forces, such as car accidents, falling injuries and heavy objects hitting (Corrigan, 2021; Mira et al., 2021). As it has a

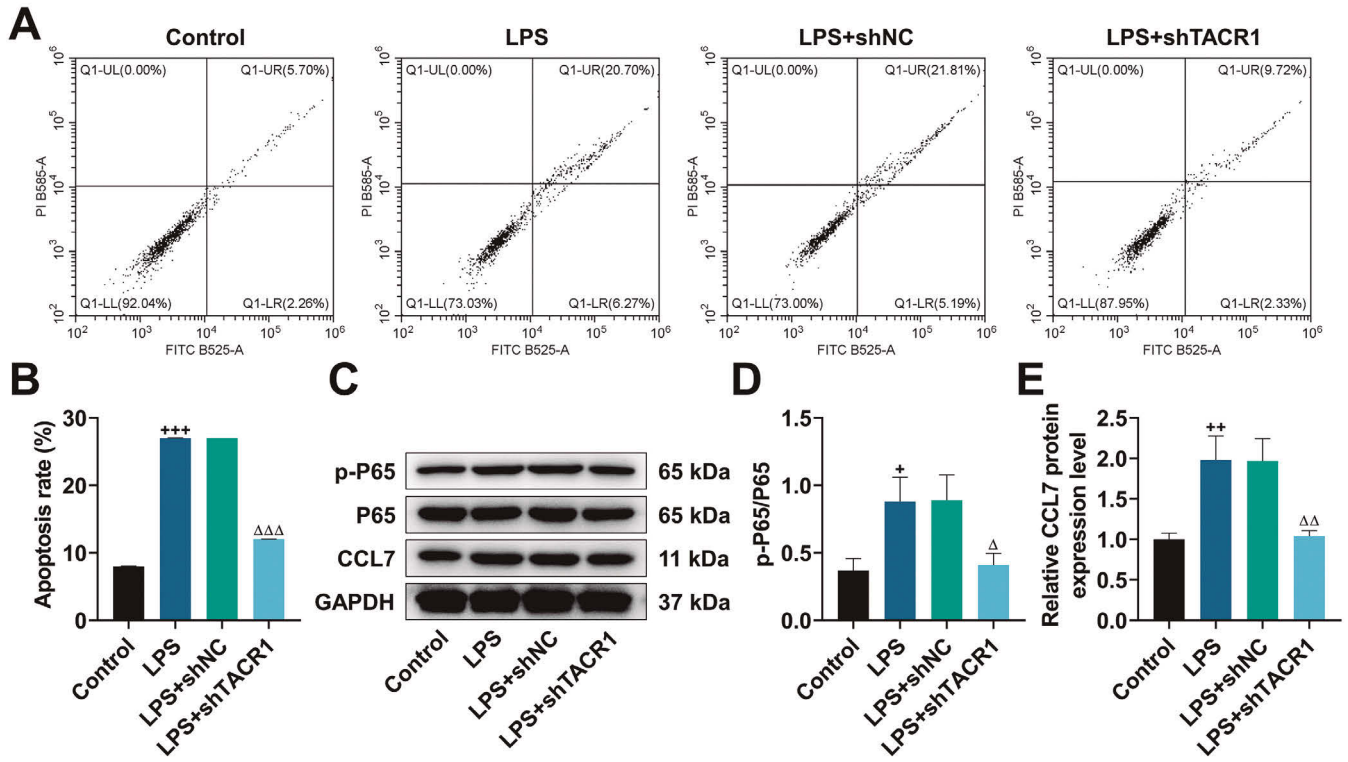


**Fig. 5.** shTACR1 reversed the effects of LPS on neuron apoptosis as well as levels of p-P65/P65 and CCL7 in microglia. **A, B.** Microglia were divided into four groups. The control group: microglia were normally cultured. The LPS group: microglia were incubated with LPS (10 ng/mL) for 4h. Based on the LPS group, microglia in LPS + shNC and LPS + shTACR1 group were transfected with shNC or shTACR1-3 for 24h. Then, the above treated microglia were co-cultured with rat neurons for 24h in 24-well transwell chambers. **A, B.** Neuron apoptosis was measured by flow cytometry assay. **C-E.** Microglia were divided into four groups. The control group: microglia were normally cultured. The LPS group: microglia were incubated with LPS (10 ng/mL) for 4h. Based on the LPS group, microglia in LPS + shNC and LPS + shTACR1 group were transfected with shNC or shTACR1-3 for 24h. **C-E.** Protein levels of p-P65, P65, CCL7 and GAPDH were determined by western blot in brain tissues of rats. GAPDH was internal reference. $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ vs. Control group. $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, $^{\Delta\Delta\Delta}P<0.001$ vs. LPS + shNC group.

high disability and death rate, TBI has been gaining more and more attention. Existing reports support that MNS plays a role in improving TBI disease (Jia et al., 2022; Zhang et al., 2022a). In the present research, we firsthand delineated that MNS improved TBI by reducing TACR1 to inhibit NF-κB and CCL7 activation in microglia.

Recently, therapeutic electrical stimulation technology has been commonly used to treat TBI disease (Park et al., 2021). The main features of TBI are consciousness loss, coma, sensory and motor dysfunction (Khellaf et al., 2019; Robinson, 2021; Scarboro and McQuillan, 2021). Notably, in TBI-induced coma rat model, MNS treatment can promote the consciousness recovery of rats (Feng et al., 2015). Consistent with this finding, our data supported that MNS treatment contributed to consciousness recovery in a TBI-induced coma rat model. In addition, there are focal necrosis and inflammatory cell infiltration in the tissue of TBI (Zhang et al., 2022b). Interestingly, MNS has the potency to improve these pathological changes in a TBI rat model (Zhao et al., 2022). In the present study, our outcomes revealed that MNS could alleviate TBI development. On the other hand, microglia are aggregated at the TBI site, which results in neuronal dysfunction (Witcher et al., 2021). An earlier study delineates that Iba1 protein is highly expressed in the progress of microglia activation (Wang et al., 2021). NeuN is the marker of neurons (Duan et al., 2016). Particularly, MNS can promote neurons regeneration after TBI (Kuo et al., 2021; Jia et al., 2022). A similar result was obtained in this study, that MNS promoted neuron production, which then improved TBI. Yet, the role of MNS in microglia activation is still unclear. We firstly found that MNS could reverse the decreased effect of TBI on Iba1 level, which indicated that MNS could improve TBI by reducing microglia activation. However, the degree of TBI injury was not assessed in this study, so the effect of MNS on TBI injury with different degrees still needs to be studied in the future.

What's more, there is a report that activated microglia can produce a large amount of pro-inflammatory factors, such as IL-1β, IL-6 and TNF-α, which is called M1 type microglia activation (Shao et al., 2022). Moreover, the appearance of M2 type microglia can promote the release of anti-inflammatory factors, such as IL-4, IL-10 and TGF-β (Colonna and Butovsky, 2017; Zhou et al., 2019). Importantly, crucial research supports that electrical stimulation in a TBI rat model can promote the production of anti-inflammatory phenotype in microglia (Park et al., 2021). The neuroinflammatory response is abnormally elevated after TBI (Chen et al., 2018). The neuroinflammatory response is abnormally elevated after TBI (Chen et al., 2018). The common markers of M1 microglia are iNOS and CD86, the usual markers of M2 microglia are Arg1 and YM1 (Colonna and Butovsky, 2017; Shao et al., 2022). These findings indicated that MNS may play a role in inflammation-related factors by regulating microglial M1/M2 polarization. Our data revealed that MNS offset the up-regulated influences of TBI on IL-1β, IL-6, TNF-α, iNOS and CD86 level as well as the down-regulated influences of TBI on IL-4, IL-10, TGF-β, Arg1 and YM1 level. These results indicated that MNS could improve TBI by promoting microglial M2 polarization and reducing neuroinflammatory response.

Reportedly, the inhibition of TACR1 can synergistically act with therapeutic electrical stimulation after TBI (Klyukin et al., 2019). Nevertheless, the effect of MNS on TACR1 level in a TBI rat model remains unknown. In the present study, it was the first outstanding finding that MNS reduced TACR1 expression in TBI rat model. Remarkably, reducing CCL7 inhibited the activation of microglia (Li et al., 2017). CCL7 can promote inflammatory reaction, which is regulated by NF-κB activation (Li et al., 2018; Liu et al., 2018). NF-κB is involved in inflammatory reaction, which is manifested as high p-P65/P65 level (Pan et al., 2021). Hence, it was an indication that NF-κB and CCL7 were closely linked with the neuroinflammatory response of microglia. Yet, there was no report about the function of MNS in regulating NF-κB and CCL7 level. Based on our data, it was the second outstanding finding that MNS reduced p-P65/P65 and CCL7 level in TBI rat model, which supported that MNS could improve TBI by decreasing NF-κB and CCL7 activation. Besides, we also need to further detected these findings by using an established model of mechanical stretch injury.

In particular, LPS-exposed microglia are usually employed to investigate the molecular mechanism of TBI disease *in vitro* (Jiang et al., 2021). LPS activated microglia (Subedi et al., 2019). Consistent with the above report, our outcomes revealed that the levels of Iba1 and F-actin were increased by LPS treatment, which indicated that microglia were activated. Moreover, TACR1 level is positively related to microglia activation (Zhu et al., 2014). Microglia can shape the fate of neurons through directly contacting with neurons (Cserép et al., 2021). Activated microglia induced by LPS can play a role in promoting neuron apoptosis (Jin et al., 2019). Herein, we found that TACR1 silencing could overturn the effects of LPS on microglia activation and neuron apoptosis. On the other hand, there are increases of pro-inflammatory factors (IL-1β, IL-6 and TNF-α) and decreases of anti-inflammatory factors (IL-4, IL-10 and TGF-β) in LPS-exposed microglia (Xu et al., 2020; Yang et al., 2020). Notably, TACR1 can inducea pro-inflammatory response (Pappa et al., 2021). Similar results were obtained in our research. Furthermore, there is an increase of M1 microglia under the action of LPS (Orihuela et al., 2016). However, there was no study about the influence of TACR1 on the polarization of M1/M2 microglia. In our study, it was the third significant finding that TACR1 silencing could reduce iNOS and CD86 level as well as elevate Arg1 and YM1 level in LPS-exposed microglia, which indicated that TACR1 was involved in the progress of microglia polarization.

Early study showed that NF-κB is overly activated after LPS stimulation (Xu et al., 2020). Especially, NF-

κB can affect CCL7 expression (Li et al., 2018). High level of CCL7 aggravates microglia-mediated inflammation response in a TBI rat model (Xue et al., 2021). This evidence indicated that the decreases of NF-κB and CCL7 level may contribute to mitigate the neuroinflammatory response of TBI. One study delineates that TACR1 can trigger cell pro-inflammatory signal by activating NF-κB (Williams et al., 2007). Hence, it was an indication that TACR1 may exert its effect on neuroinflammatory response by regulating NF-κB and CCL7 activation. Our data verified that TACR1 silencing could reduce p-P65/P65 and CCL7 level in LPS-exposed microglia, which then lessened neuroinflammatory response. For these findings, relevant animal experiments will be performed in the future.

Taken together, our research illustrated an underlying mechanism explanation for the effect of MNS on TBI disease. Our data demonstrated that MNS improved TBI by reducing TACR1 to inhibit NF-κB and CCL7 activation in microglia. In addition, our studies provided a theoretical basis for the mechanism of MNS in TBI treatment. TACR1, NF-κB and CCL7 had the potential value to serve as critical molecular targets to treat TBI disease.

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*Statement of Ethics.* Adult and newborn Sprague-Dawley rats were obtained from Hangzhou Medical College (Zhejiang, China). Rats were kept in a 12 h light-dark cycle room (25°C room temperature and 47% humidity). Rats could freely gain standard water and food. All experimental operations on rats were performed in accordance with the China Council on Animal Care and Use guidelines and were approved by the Ethics Committee of Zhejiang Baiyue Biotech Co., Ltd for Experimental Animals Welfare (ZJBYLA-IACUC-20220815).

*Conflict of Interest Statement.* The authors declare no conflicts of interest.

*Author Contributions.* Substantial contributions to conception and design: Fan Sun and Min Shi; Data acquisition, data analysis and interpretation: Xiaodong Li, Xiaowei Wan1, Yali Ou, Xuesong Li and Wenbing Liu; Drafting the article or critically revising it for important intellectual content: Fan Sun and Min Shi; Final approval of the version to be published: All authors; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: Fan Sun, Min Shi, Xiaodong Li, Xiaowei Wan1, Yali Ou, Xuesong Li, Wenbing Liu

*Data Availability Statement.* The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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