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Transcutaneous occipital nerve stimulation alleviated migraine related pain by regulating synaptic plasticity and CGRP expression in the periaqueductal gray of male rats

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Abstract

Background Clinical observations have shown that transcutaneous occipital nerve stimulation (tONS) is effective in treating migraine. However, the underlying mechanisms are poorly understood. This study employs a rodent model to investigate the therapeutic effects of tONS on migraine-related pain and to explore potential mechanisms.

Methods The SD rats were used to establish the migraine model by repeated epidural infusions of inflammatory soup (IS). Modified bilateral electrodes were attached noninvasively for tONS treatments. Periorbital mechanical thresholds were assessed using von-Frey filaments, and other pain-related nociceptive behaviors were analyzed through video recordings. The expressions of c-Fos, synaptophysin (Syp) and calcitonin gene-related peptide (CGRP) in the trigeminal nucleus caudalis (TNC) and/or periaqueductal gray (PAG) area were measured by immunofluorescence and western blotting analyses. The excitatory synaptic transmission in the PAG was detected by whole-cell patch-clamp recording among migraine rats.

Results The reduction in periorbital mechanical thresholds induced by repeated IS infusions was partially reversed by tONS treatments in migraine rats. Other pain-related behaviors, including exploration, rest, and unilateral grooming, consistently improved following tONS treatment. The TNC and PAG area were activated after IS modeling, and the CGRP expressions in the PAG significantly decreased after tONS treatments. tONS could inhibit the enhanced excitatory synaptic transmission in the PAG of migraine rats.

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Conclusions Our findings suggest that tONS has therapeutic potential in treating migraine, with the PAG excitability and CGRP expression playing a role in its mechanisms of action. tONS may represent a promising non-invasive neuromodulation approach for the management of migraine in the future.

Keywords Neuromodulation, Migraine, Nociceptive behaviors, Synaptic plasticity, Neuropeptide

Background

Migraine is a recurrent disabling disorder. The latest report on the global burden of disease places migraine in second place, where it is responsible for 5.6% of all years lived with disability (YLDs) in the world. Furthermore, migraine is the top cause of YLDs in the age group 15–49 years [1, 2]. Our previous nationwide population-based headache survey in the mainland of China found a one-year migraine prevalence of 9.3%, resulting in severe burdens to patients and society [3]. The mechanisms underlying migraine are not fully understood. Currently, the most widely accepted cause is activation of the trigeminovascular pathway [4]. This pathway innervates several pain-sensitive intracranial structures, and most of these areas are dominated by A and C fibers from the ocular branches of the trigeminal nerves and the upper cervical medullary roots (C1-C3). These afferent sensory fibers converge on secondary neurons within the trigeminal cervical complex (TCC), forming synaptic connections. The TCC extends from the trigeminal nucleus caudalis (TNC) to the segments of the C2–C3, and its integration and transmission functions play an important role in the pathogenesis of migraine [5, 6]. Clinically, migraine attacks are often accompanied by discomfort in the posterior neck and trapezius muscle regions, and some studies have shown that up to 75% of patients had neck pain during migraine attacks [7, 8]. All of these findings provide evidence of the participation of the TCC in the pathogenesis of migraine. Except the TCC, the periaqueductal gray (PAG) area also plays a major role in the modulation and perception of pain [9]. Through both ascending and descending projections, the PAG could lessen or augment pain perception due to its bidirectional manner [10]. Previous report showed that the stimulation of the PAG before surgery resulted in the decreased anesthetic requirements. Moreover. the PAG has been implicated in the pathophysiology of migraine, possibly being the "generator" sites for migraine attacks [11].

Nowadays, neuromodulation, especially electric neurostimulation (ENS), as a classical non-pharmacological approach is drawing much attention due to the limitations of traditional pharmacological methods, such as contraindications and intolerance [12]. Since Pattle et al. first proposed the concept of ENS in the field of pain research, it has developed into a relatively mature intervention mode [13]. Considering the important role of the TCC in migraine pathogenesis, ENS stimulation is often applied to the occipital nerves as branches of the C2 nerve [14]. Several randomized controlled studies have been conducted on occipital nerve stimulation (ONS), in which both occipital nerves were stimulated in patients after electrodes implantation. The results confirmed the efficacy of ONS and suggested a promising future for ONS in the treatment of several types of primary headache and, especially for chronic migraine [15]. However, ONS is an invasive method that requires electrodes implantation which can lead to complications as well as high risks associated with surgery [16, 17]. Additionally, patient with migraine who experience mild headache intensities usually make up a significant proportion in clinical practice, for whom invasive ONS is obviously unsuitable. Thus, transcutaneous electrical neurostimulation (TENS) treatment, a non-invasive peripheral nerve electrical stimulation, is gradually coming under the global spotlight. TENS has the advantages of simple operation, a high level of safety, and a high degree of acceptance [18]. Several clinical studies have shown that supraorbital TENS is effective for both acute and preventive treatment of migraine [19, 20].

Previously, our team conducted a prospective randomized controlled trial of the single use of transcutaneous ONS (tONS) for prophylactic treatment in migraine patients. The results illustrated that tONS was safe, effective, and well-tolerated in migraine patients with frequent attacks, especially in terms of the 50% response rate and reduction of headache intensity [21]. However, the influence of patients' expectation could not be ruled out, and there is still a lack of research on the mechanisms of tONS. Thus, this study aimed to further explore the therapeutic effects of tONS and tentatively explore possible mechanisms of action through changes in animal behavior and related indicators using a rodent model induced by repeated epidural inflammatory soup (IS) stimulations. We found that the periorbital mechanical thresholds were decreased by repeated IS infusions and were partially reversed after tONS treatments. Besides, tONS significantly alleviated nociceptive behaviors induced by repetitive IS infusions. The expressions of c-Fos as well as calcitonin gene-related peptide (CGRP) were elevated after modeling and decreased after tONS in the TNC and PAG area. Furthermore, tONS inhibited the enhanced excitatory synaptic transmission in the PAG of IS rats. These results indicated that tONS could alleviate migraine-related pain by regulating synaptic plasticity and CGRP expression in the PAG. It is a supplement to our previous clinical research and could lay an

experimental foundation for the development of a new therapeutic approach to migraine in the future.

Methods

Animals

The adult male Sprague–Dawley (SD) rats (8–9 weeks old) were purchased from Beijing SBV Biotechnology Company and housed individually in a temperature-(23 ± 2℃) and humidity (50 ± 10%)-controlled environment with free access to food and water for seven days. A standard 12-/12-h light/dark cycle was maintained. This study was approved by the Committee on Animal Use for Research and Education of the Laboratory Animals Centre at the Chinese People's Liberation Army (PLA) General Hospital and followed the ethical guidelines for the study of pain in conscious animals (registration number: S2021-349-02).

Cranial surgical procedure

A cannula was implanted in each rat for dural infusion, as described previously [22, 23]. Briefly, rats were anesthetized to a deep surgical plane with 3% pentobarbital sodium (2 mL/kg, i.p.) and placed in a stereotactic frame. An incision was made on the dorsal surface of the scalp to expose the skull followed by a 1-mm diameter craniotomy in the left frontal bone to expose the dura adjacent to the superior sagittal sinus (1.5 mm lateral to the midline and 1.5 mm posterior to bregma). A plastic cap with a stainless-steel inner cannula was implanted in the drilled cranial window for delivery of IS or normal saline (NS). After cannula surgery, the rats were housed separately for the subsequent experiments. The rats were allowed to recover for 7 days (Day -7–-1) to ensure return of their sensory thresholds to presurgical baselines.

Experimental design and groups

All rats received 14 days of IS/NS infusion modeling (Day 0–13) followed by five tONS treatments (Day 14–18). The rats were divided into four groups according to modeling (IS or NS) and tONS treatment (true or sham), namely, IS + True, IS + Sham, NS + True, and NS + Sham. The IS comprised 1 mM histamine, 1 mM 5-HT, 1 mM bradykinin, and 0.1 mM prostaglandin E2 [24, 25]. In awake rats, ten microliters of IS or NS were administered during 5 min.

tONS treatment was conducted since Day 14 to Day 18. Rats were anesthetized by 2% isoflurane inhalation and placed in a prone position. The position of the occipital nerves was identified by the bilateral occipital notches and modified 1.5 cm*1.5 cm self-adhesive electrodes were firmly attached to cover the skin surface. Rats were electrically stimulated using a HANS-200 A machine (JiSheng Medical Technology Ltd Co, China) for 30 min daily for five days [26]. The true tONS stimulation groups received tONS at 2/100 Hz with an intensity of 4 mA, while the sham groups were treated in the same manner but with no current output (0 mA) [26, 27].

Periorbital mechanical threshold test

Nociceptive thresholds were measured daily before IS/NS injection by perpendicular application of von- Frey filaments (0.4–26 g, North Coast Medical Co., Ltd., USA) to the periorbital region until it buckled slightly, and maintained for 3 to 6 s or until the rat responded positively [22]. Thus, the data of Day 0 were used as baseline when rats did not receive any injection. Positive responses included withdrawing the face from the monofilament, escape, attack, or asymmetrical face grooming. The thresholds were determined by the 'up-down' method [28]. Rats that did not respond to the 26 g stimulus were recorded as having a 26 g threshold.

Video-recorded behavioral analysis

The rats were videotaped for 900 s on alternate days after beginning IS/NS stimulation. The amount of time (in seconds) spent in exploratory and rest behaviors and unilateral and bilateral grooming were recorded [29]. The unilateral grooming behavior means the ipsilateral hindpaw facial grooming, without distinguishing whether it was on the same side as the injection [29]. The videos were analyzed by two blinded researchers.

Immunofluorescence staining and counting

All target tissues were collected 24–28 h after the last operations. The brains were perfused with 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) and 4% paraformaldehyde, removed, and dehydrated with graded sucrose solutions before sectioning (10 μm sections). Sections were blocked with 10% goat serum for 30 min and incubated with anti- CGRP (1:200; ab36001, Abcam, USA) and anti-c-Fos (1:1000; ab190289, Abcam, USA) primary antibodies for 12 h, followed by Alexa 488-conjugated anti-rabbit IgG (1:1000; Thermo Fisher, USA) for 2 h. The sections were stained with 4,6-diamidino-2-phenylindole (DAPI) for 30 s and washed out with PBS. The coverslip was sealed with an anti-fluorescent quencher, and evaluated by fluorescence microscopy. Three randomly selected images at 40 times magnification were recorded for the TNC and PAG regions (identified using a rat brain atlas: TNC, also known as Sp5c in atlas: Interaural – 5.52 mm and Bregma-14.52 mm; PAG: Interaural 0.84 mm and Bregma – 8.16 mm). Immunolabeling of cells was analyzed by Image J and CaseViewer software without specifying rat identities with the average integrated density of the three images representing the final results.

Western blotting analysis

Protein preparation and western blotting analysis were performed according to a previous method [25, 30]. Briefly, the bilateral TNC and PAG brain tissues were collected and homogenized in ice-cold lysis buffer containing 0.1% phenylmethylsulfonyl fluoride. The homogenates were centrifuged (12 000 rpm, 10 min) and protein concentrations in the supernatants were measured using a bicinchoninic acid protein assay kit (C2026; Servicebio Technology). Proteins (40 μg) were separated on SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline containing Tween 20 (TBST) for 30 min and probed with the following antibodies: anti-CGRP (1:1000, cell signaling technology, #14959, USA), anti-synaptophysin (1:10000, Abcam, ab32127, USA), anti-β-actin (1:2000, Servicebio, GB12001, China), anti-GAPDH (1:1000, Servicebio, GB12002, China). After three washes in TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000; GB23302; Servicebio Technology) for 30 min. The antibody-reactive bands were visualized using enhanced chemiluminescence detection reagents (G204; Servicebio Technology) and a gel imaging system (Alpha Innotech).

Brain slices preparation

Coronal brain slices (300 μm) at the level of the PAG were prepared using standard methods [30, 31]. Briefly, rats were deeply anesthetized with 5% isoflurane and transcardially perfused with cold cutting solution containing (in mM) 2.5 KCl, 0.5 $CaCl_2$, 10 $MgSO_4$, 1.25 NaH_2PO_4, 2 thiourea, 3 sodium pyruvate, 92 N-methyl-D-glucamine, 20 N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), 25 D-glucose, 5 L-ascorbic acid, and 30 $NaHCO_3$ (pH 7.35–7.40). Then the rats were sacrificed by decapitation and the whole brain was removed quickly from the skull and submerged in the oxygenated (95% O_2 and 5% CO_2) ice-cold cutting solution. The whole brain tissue was cooled for short time before trimmed as the proper part to glue onto the microslicer (VT1200S Vibratome, Leica, Germany). The coronal brain slices containing the PAG were cut, and then incubated in a submerged recovery chamber with artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 2 $MgSO_4$, 1 NaH_2PO_4, 2 $CaCl_2$, 25 $NaHCO_3$, and 10 D-glucose at room temperature for 1 h. The ACSF was continuously aerated with a mixture of 95% O_2 and 5% CO_2.

Whole-cell patch-clamp recording

Whole-cell recordings were performed in a recording chamber on the stage of an Axioskop 2FS microscope with infrared differential interference contrast optics for visualization. sEPSCs were recorded with HEKA EP10 amplifier (HEKA, Germany). The recording pipettes (3–5MΩ) were filled with the solution containing (in mM) 112 Cs-Gluconate, 5 TEA-Cl, 3.7 NaCl, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na_3-GTP and 5 QX-314, which adjusted to pH 7.2 with CsOH and had osmolality of 290 mOsmol. Picrotoxin (PTX,100 μM, Tocris, UK) was presented to block the $GABA_A$ receptor-mediated inhibitory synaptic currents. Data were discarded if access resistance changed > 15% during an experiment. Data were filtered at 1 kHz, and digitized at 10 kHz.

Statistical analysis

SPSS 27.0 software (IBM Corp., USA) was used for statistical analysis and Prism 9.5 software was used for mapping. Whole-cell patch-clamp data were collected and analyzed by Clampex 9.0 and Clampfit 9.0 software (Molecular Devices). sEPSCs were analyzed by an event detection program (Mini Analysis Program; Synaptosoft, Inc., Decatur, GA). Two-way repeated measures ANOVA was used for analysis of nociceptive thresholds and behavioral tests, while immunofluorescence results were analyzed by two-way ANOVA, followed by Bonferroni for post hoc tests. Data distribution was analyzed by Shapiro-Wilk's test, homogeneity of variance by Levene's method, and the Greenhouse-Geisser method was used to correct for interaction between two factors. If interaction between two factors occurred, the effects were analyzed separately, otherwise, the main effect was analyzed and pairwise comparison was made as required. $P < 0.05$ was considered statistically significant, and data were presented as mean ± standard error of the mean (SEM).

Results

Periorbital nociceptive thresholds were decreased by repeated IS infusions and were partially reversed after tONS treatments

The detailed flow chart of study is shown in Fig. 1. Rats received infusions of IS (IS + True/Sham groups) or NS (NS + True/Sham groups) ($n = 10$ rats/group, 40 rats in total) between Day 0 and Day 13. The periorbital mechanical thresholds did not differ among the four groups at baseline (Day 0), while the mechanical thresholds of the IS groups decreased significantly, remaining stable between Days 11 and 13 compared with the NS groups (IS + Sham vs. NS + Sham: Day 11: 0.88 ± 0.17 g vs. 22.70 ± 1.68 g, $F_{(3, 27)} = 159.36$, $P < 0.01$; Day 13: 0.88 ± 0.18 g vs. 24.90 ± 1.10 g, $F_{(3, 27)} = 286.63$, $P < 0.01$; Fig. 2). The tONS (true or sham) treatments were given from Day 14 to Day 18 and the rats were sacrificed on Day 19. It was found that, in the NS groups, the nociceptive thresholds between Day 15 and Day 19 did not change significantly after tONS stimulation ($P = 0.094$). In contrast, compared with the IS + Sham group, the periorbital nociceptive thresholds of the IS + True group

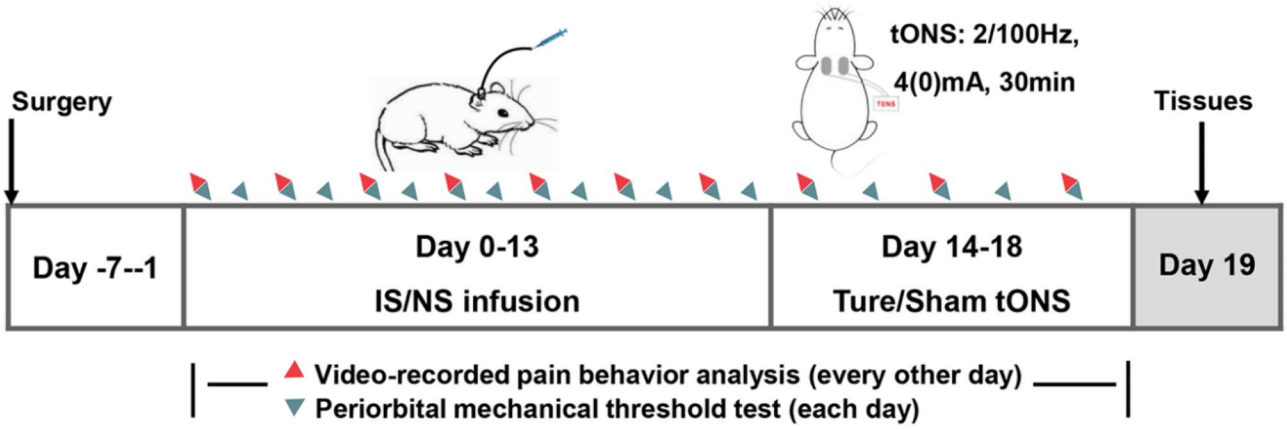

Fig. 1 Flow chart of the experimental outline. Rats received 14 days of 10 μL IS/NS modeling (Day 0–13) followed by five-time treatments with tONS (Day 14–18). Periorbital mechanical thresholds were measured daily and video-based behavioral pain assessments were conducted on alternate days (Day 0–18). Rats were sacrificed on Day 19 for tissue harvesting. IS: inflammatory soup; NS: normal saline. tONS: transcutaneous occipital nerve stimulation. tONS parameters: True: 2/100 Hz, 4 mA, 30 min; Sham: 2/100 Hz, 0 mA, 30 min

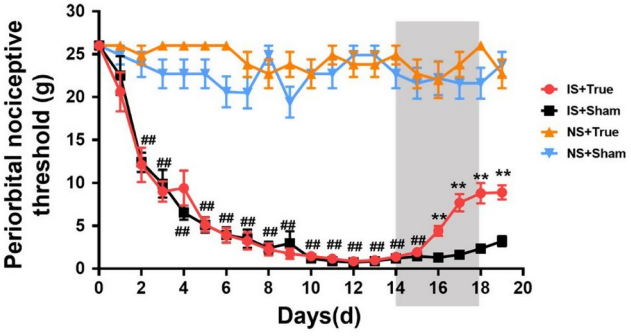

Fig. 2 Periorbital nociceptive thresholds in rats after different modeling and tONS treatments. The nociceptive thresholds in rats in the IS + Sham groups were significantly lower than those in the NS + Sham groups from Days 11 to Day 13 and thereafter remained stable ($n = 10$ rats/group). After true tONS treatment, the nociceptive threshold was observed to be significantly higher in the IS + True groups than in the IS + Sham group on Day 16 and lasted until the final test. The shadow represents the period of tONS treatments (Day 14 to Day 18). ## $P < 0.01$, IS + Sham vs. NS + Sham; ** $P < 0.01$, IS + True vs. IS + Sham, error bars represent SEM

were significantly raised on Day 16, lasting until Day 19 (IS + True vs. IS + Sham: Day 16: 4.40 ± 0.58 g vs. 1.30 ± 0.37 g, $F_{(3, 27)} = 64.93$, $P < 0.01$; Day 19: 8.90 ± 0.82 g vs. 3.20 ± 0.59 g, $F_{(3, 27)} = 68.37$, $P < 0.01$; Fig. 2).

tONS alleviated video-recorded nociceptive behaviors induced by repetitive IS infusions

Video-recorded nociceptive behaviors were investigated on alternate days to evaluate pain reactions before and after tONS treatment ($n = 10$ rats/group, Fig. 3). The time spent exploring in the IS groups was gradually reduced after Day 8 and remained constant until tONS treatment (IS + Sham vs. NS + Sham: Day 8: 443.40 ± 22.34 s vs. 640.20 ± 19.27 s, $F_{(3, 27)} = 14.72$, $P < 0.01$; Day 12: 408.30 ± 30.65 s vs. 618.00 ± 20.96 s, $F_{(3, 27)} = 20.15$, $P < 0.01$; Fig. 3A). After true tONS treatments, the time spent in exploratory behavior in the IS + True group was significantly longer than that in the IS + Sham group on Day 18 (IS + True vs. IS + Sham: Day 18: 593.80 ± 15.42 s vs. 479.10 ± 13.41 s, $F_{(3, 27)} = 14.55$, $P < 0.01$, Fig. 3A).

From Day 8, the resting times in the IS groups increased gradually compared with the NS groups and continued until tONS treatment (IS + Sham vs. NS + Sham: Day 8: 147.50 ± 20.19 s vs. 55.90 ± 10.53 s, $F_{(3, 27)} = 7.50$, $P = 0.049$; Day 12: 190.50 ± 19.03 s vs. 46.40 ± 11.41 s, $F_{(3, 27)} = 37.04$, $P < 0.01$; Fig. 3B). The resting times in the IS + True group were significantly reduced on Day 16 and 18 compared with the IS + Sham group (IS + True vs. IS + Sham: Day 16: 111.90 ± 11.04 s vs. 162.30 ± 9.95 s, $F_{(3, 27)} = 38.90$, $P = 0.039$; Day 18: 73.30 ± 10.38 s vs. 135.00 ± 10.18 s, $F_{(3, 27)} = 15.38$, $P = 0.014$; Fig. 3B).

The unilateral grooming times were significantly increased in the IS groups from Day 8 compared with the NS groups and remained constant until treatment (IS + Sham vs. NS + Sham: Day 8: 156.10 ± 12.85 s vs. 66.60 ± 9.50 s, $F_{(3, 27)} = 32.93$, $P < 0.01$; Day 12: 193.70 ± 13.32 s vs. 83.80 ± 6.98 s, $F_{(3, 27)} = 56.01$, $P < 0.01$; Fig. 3C). After tONS treatment, the unilateral grooming times decreased gradually in the IS + True group, with significant differences between the IS + True and IS + Sham groups on Days 16 and Day 18 (IS + True vs. IS + Sham: Day 16: 101.50 ± 11.33 s vs. 169.90 ± 12.64 s, $F_{(3, 27)} = 33.94$, $P < 0.01$; Day 18: 86.80 ± 6.35 s vs. 156.90 ± 12.70 s, $F_{(3, 27)} = 34.67$, $P < 0.01$; Fig. 3C).

Finally, we assessed the bilateral grooming times in the different groups. No difference was observed between the IS and NS groups during the modeling period, and no significant differences were found in the main effects of time and intervention (IS + Sham vs. NS + Sham: Day 8: 26.80 ± 6.65 s vs. 29.40 ± 4.61 s, $F_{(3, 27)} = 0.21$, $P = 0.887$; Day 12: 31.00 ± 6.20 s vs. 26.80 ± 5.75 s, $F_{(3, 27)} = 0.25$,

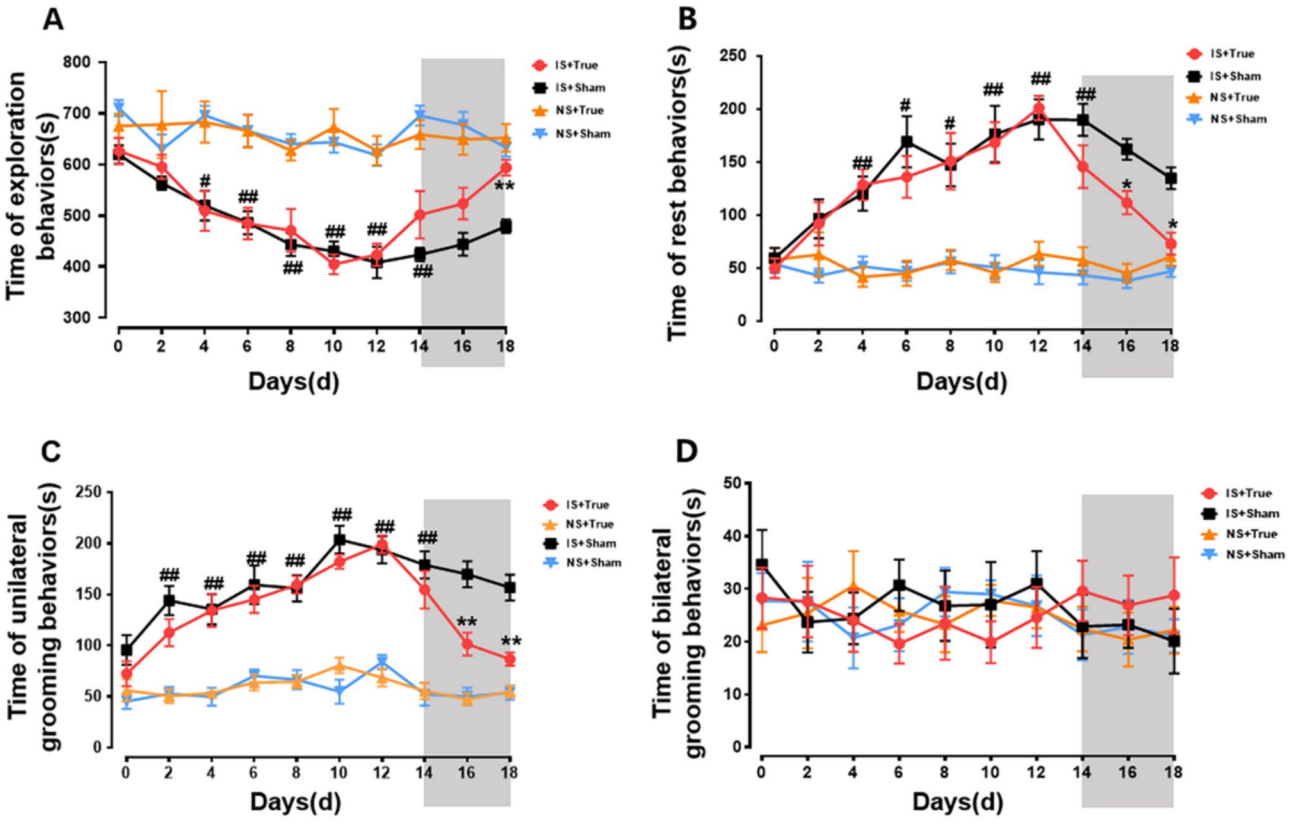

Fig. 3 Video-recorded nociceptive behaviors were induced by IS and ameliorated by tONS treatment. (**A**) The times spent on exploratory behaviors in the IS groups were significantly increased on Day 18 in rats receiving true tONS stimulation compared with the sham group ($n = 10$ rats/group). (**B**) The times spent resting in the IS groups were significantly reduced on Day 16 and Day 18 in rats receiving true tONS stimulation compared with the sham group ($n = 10$ rats/group). (**C**) The times spent on unilateral grooming in the IS groups were significantly reduced on Day 16 and Day 18 in rats receiving true tONS stimulation compared with the sham group ($n = 10$ rats/group). (**D**) Times spent on bilateral grooming in rats in the four groups. There were no significant differences observed with either modeling or tONS treatments ($n = 10$ rats/group). The shadow represents the period of tONS treatments (Day 14 to Day 18). # $P < 0.05$, ## $P < 0.01$, IS + Sham vs. NS + Sham; * $P < 0.05$, ** $P < 0.01$, IS + True vs. IS + Sham, error bars represent SEM

$P = 0.858$, Fig. 3D). The results were similar after tONS treatment, with no significant changes from the modeling period.

tONS influenced the activations of the TNC and PAG in a rat model of migraine

The c-Fos expression in the TNC was significantly higher in the IS + Sham group than that in the NS + Sham group (85.19 ± 4.15 vs. 38.47 ± 2.12; $n = 6$ rats/group; $F_{(1, 20)} = 59.03$, $P < 0.01$; Fig. 4A-C). The positive cells of c-Fos protein in the IS + True group was significantly lower than in the IS + Sham group (51.86 ± 4.78 vs. 85.19 ± 4.15, $F_{(1, 20)} = 30.05$, $P < 0.01$). There was no significant difference between the NS + True and NS + Sham groups ($P = 0.702$) as well as IS + True and NS + True groups ($P = 0.085$). Moreover, in the PAG, the positive cells of c-Fos protein was significantly higher in the IS + Sham group than in the NS + Sham group, and was lower in the IS + True group (IS + Sham vs. NS + Sham: 73.67 ± 5.26 vs. 30.00 ± 2.06, $F_{(1, 20)} = 52.66$, $P < 0.01$); IS + Sham vs. IS + True:73.67 ± 5.26 vs. 50.50 ± 4.44, $F_{(1, 20)} = 14.82$, $P < 0.01$; IS + True vs. NS + True: 50.50 ± 4.44 vs. 40.83 ± 5.41, $F_{(1, 20)} = 4.51$, $P = 0.046$; $n = 6$ rats/group; Fig. 5A-C).

tONS alleviated increased CGRP expression in the PAG

Both immunofluorescence staining and western blotting tests showed that the expression of CGRP in the PAG increased among rats suffering the repeated IS infusions (immunofluorescence staining: IS + Sham group vs. NS + Sham group: 64.50 ± 2.17 vs. 20.94 ± 1.17; $n = 6$ rats/group; $F_{(1, 20)} = 29.83$, $P < 0.01$; Fig. 6A; western blotting tests: IS + Sham group vs. NS + Sham group: 1.26 ± 0.06 vs. 0.58 ± 0.17; $n = 5$ rats/group, $F_{(1, 16)} = 22.01$, $P < 0.01$; Fig. 6B). The following tONS treatments significantly reduced the expression of CGRP within IS groups. The number of CGRP-positive cells in the IS + True group was significantly reduced compared with the IS + Sham group (38.06 ± 2.68 vs. 64.50 ± 2.17; $n = 6$ rats/group; $F_{(1, 20)} = 86.20$, $P < 0.01$; Fig. 6A), with no significant difference between the NS + True and NS + Sham groups ($P = 0.591$). The western blotting tests also found

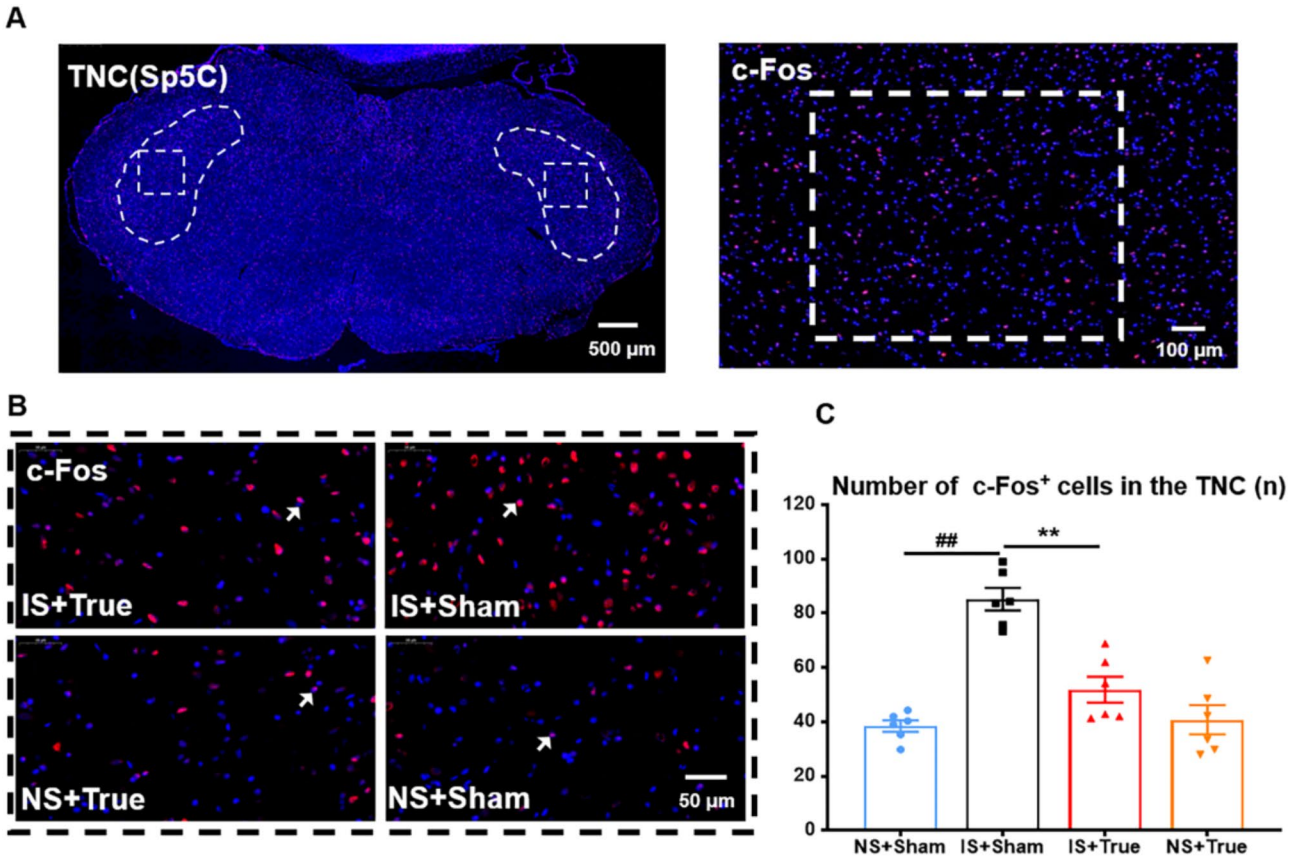

Fig. 4 Expression of c-Fos in the TNC after modeling and tONS treatment among four groups. (**A**) Representative images of c-Fos (red) immunofluorescence staining in the location of the TNC (left). Enlarged area for statistical analysis (right). (**B**) Representative images of c-Fos (red) and DAPI (blue) immunofluorescence staining among different groups within the TNC. White arrow showed the merged c-Fos-positive and DAPI-positive cells. (**C**) The numbers of c-Fos-positive neurons in the TNC increased significantly after repeated IS infusions compared with the NS group. Significantly reduced cell numbers were observed in the IS + True group compared with the sham tONS group ($n = 6$ rats/group). ## $P < 0.01$, IS + Sham vs. NS + Sham; ** $P < 0.01$, IS + True vs. IS + Sham; error bars represent SEM. Sp5c, caudal part of the spinal trigeminal nucleus

significant attenuated in CGRP levels between IS + True and IS + Sham groups (0.89 ± 0.06 vs. 1.26 ± 0.06; $n = 5$ rats/group; $F_{(1, 16)} = 6.44$, $P = 0.022$; Fig. 6B). Additionally, there was no significant difference between the IS + True and NS + True groups ($P = 0.089$).

tONS inhibited the enhanced excitatory synaptic transmission in the PAG of IS rats

The schematic diagram and representative recording diagram for spontaneous excitatory postsynaptic currents (sEPSCs) in the PAG were shown in Fig. 7A-C. The frequency of sEPSCs was significantly decreased in IS + True rats compared with IS + Sham rats (1.63 ± 0.30 vs. 2.91 ± 0.4; $n_{(IS+True)} = 9$ neurons/4 rats; $n_{(IS+Sham)} = 8$ neurons/4 rats; $P = 0.022$; Fig. 7D left). While, the amplitude of sEPSCs did not show significant differences between two groups (8.28 ± 0.53 vs. 8.24 ± 0.49; $n_{(IS+True)} = 9$ neurons/4 rats, $n_{(IS+Sham)} = 8$ neurons/4 rats, $P = 0.84$; Fig. 7D right).

To further test whether IS-modelling and tONS affects synaptic plasticity in the PAG, the expression of the synapse-associated proteins synaptophysin (Syp) was detected (Fig. 8). A western blotting analysis showed that the expression levels of Syp was significantly lower in the IS + True group than in the IS + Sham group (0.60 ± 0.07 vs. 1.01 ± 0.19; $n = 5$ rats/group; $F_{(1, 16)} = 5.23$, $P = 0.036$). There was no significant difference between the IS + True and NS + True groups ($P = 0.204$).

Discussion

The current study used a rodent model established by repeated dural IS stimulations mimic migraine-like pain and related nociceptive behaviors, using NS application as control. The tONS treatments as one of non-invasive neuromodulation therapies were used among IS/NS rats. The results showed that the activations of the TNC and PAG are involved into the pathological conditions of migraine, and influenced by tONS treatments. We found that the IS rats who received true tONS treatments showed increased nociceptive thresholds and duration of exploratory behavior, together with shorter rests and unilateral grooming times compared with the sham tONS

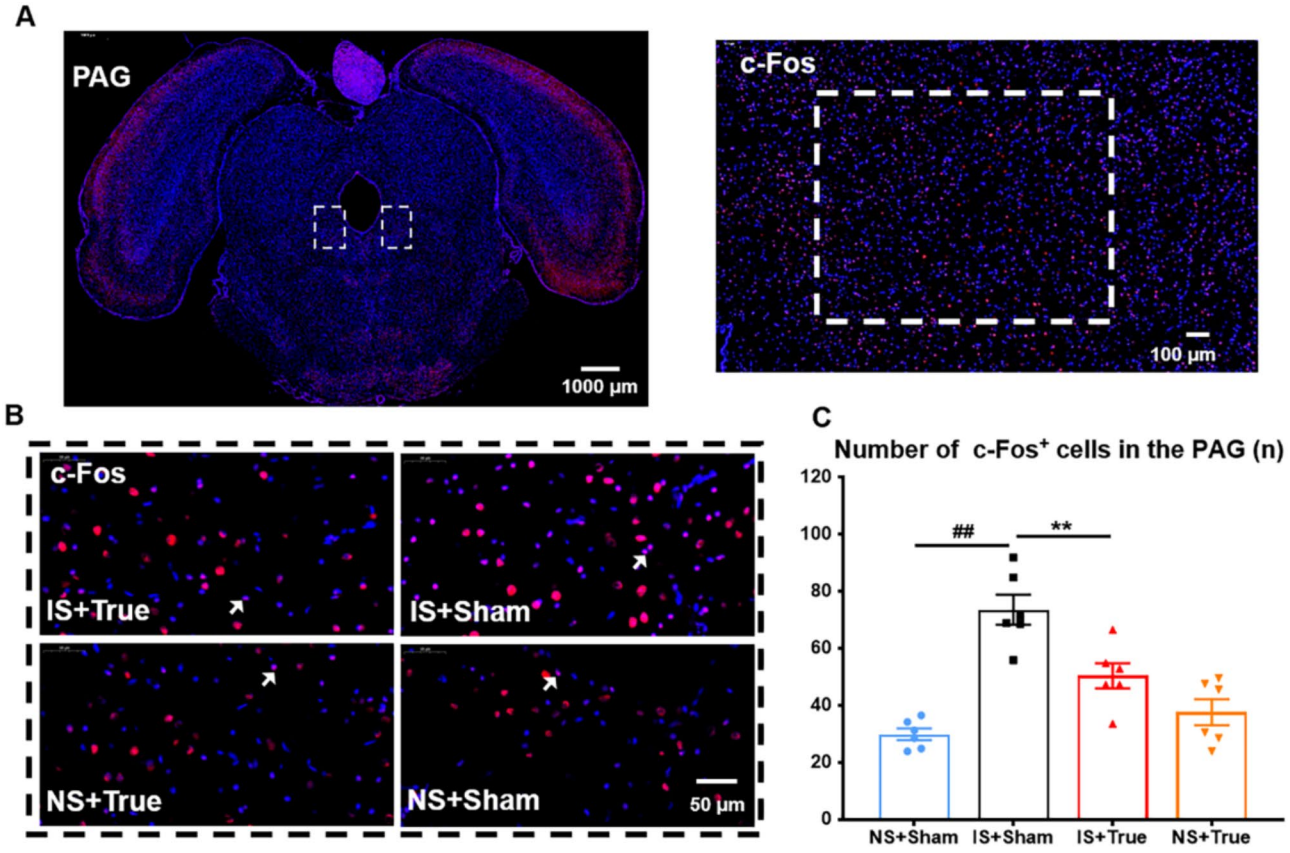

Fig. 5 Expression of c-Fos in the PAG after modeling and tONS treatment among four groups. (**A**) Representative images of c-Fos (red) immunofluorescence staining in the location of the PAG area (left). Enlarged area for statistical analysis (right). (**B**) Representative images of c-Fos (red) and DAPI (blue) immunofluorescence staining among different groups within the PAG. White arrow showed the merged c-Fos-positive and DAPI-positive cells. (**C**) The numbers of c-Fos-positive neurons in the PAG increased significantly after repeated IS infusions compared with the NS group. Significantly reduced cell numbers were observed in the IS + True group compared with the sham tONS group ($n = 6$ rats/group). ## $P < 0.01$, IS + Sham vs. NS + Sham; ** $P < 0.01$, IS + True vs. IS + Sham; error bars represent SEM

groups. The integrated data of biochemical and physiological tests indicated that tONS treatments inhibited the enhanced excitatory synaptic transmission of IS rats as well as attenuated the increased expression of CGRP in the PAG.

The therapeutic effect of tONS in migraine

The classical rodent model of repeated infusions of IS were used in the present study to activate the trigeminal neurovascular system to mimic trigeminal sensitization in the pathogenesis of migraine, as demonstrated in previous studies [23, 25, 32]. The reductions in the nociceptive threshold were observed on Day 2 compared with the NS groups, with the differences between the groups increasing in correspondence with the days of IS stimulations. In addition, the periorbital nociceptive thresholds in the IS groups were lower between Day 11 to Day 13, and were sustained, further laying a foundation for confirming the effect of tONS. Besides, the behavioral changes observed in the rats were similar to those seen in migraine patients who avoid physical activity due to painful headache or who rest because the headache intensity is aggravated by daily activities. However, the results of bilateral grooming times did not reach significance; this is consistent with the findings of Melo-Carrillo and colleagues who suggested that unilateral grooming may complement the unilateral headache that is common in migraine patients [29]. Therefore, the changes in the periorbital nociceptive threshold and behavioral indicators suggest the feasibility and reliability of applied rat migraine model, which may approximate the incidence of clinical experience.

The parameters chosen for tONS based on the Jisheng Han and colleagues' work, which showed the frequency of 2/100Hz produced simultaneous release of four different types of opioid peptides (namely enkephalin, beta-endorphin, endomorphin and dynorphin), resulting in a maximal therapeutic effect in several chronic pain patterns [26]. The measurements from Day 16 to Day 18 showed significant increases in the periorbital nociceptive threshold of rats in the IS groups after true tONS treatments. Correspondingly, the times spent in exploratory behavior increased together with reductions in the

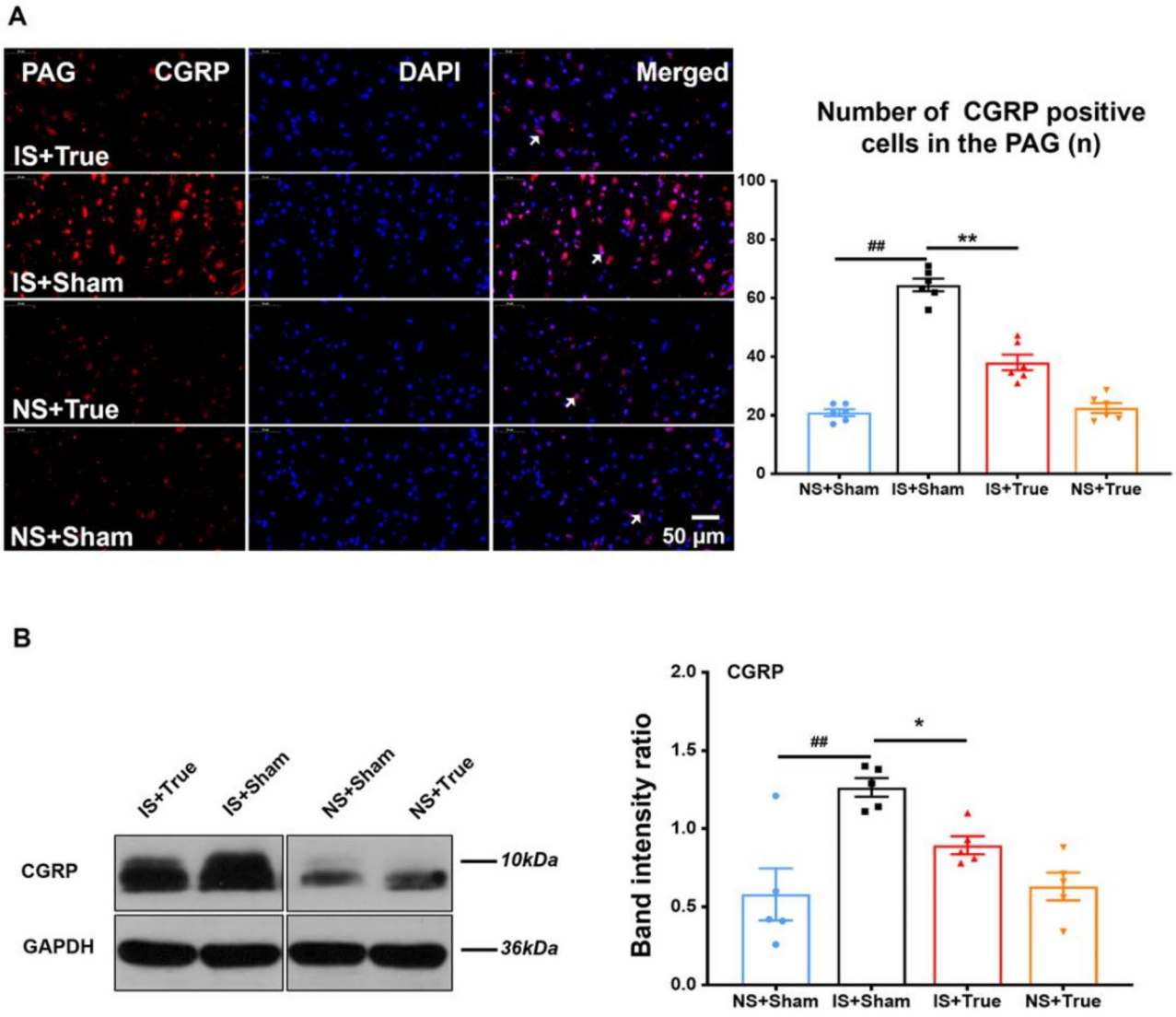

Fig. 6 tONS treatment alleviated CGRP expressions in the PAG of IS rats. (**A**) Representative images of immunofluorescence staining showing CGRP-labeled cells (red) in the PAG (left). The numbers of CGRP-positive neurons in the PAG increased significantly after repeated IS injections compared with the NS group (right). Significantly reduced cell numbers were observed in the IS + True group compared with the sham tONS treatment ($n = 6$ rats/group, right). (**B**) Typical western blot images of CGRP from the PAG (left). The band intensity ratio of CGRP in the PAG increased significantly after repeated IS injections compared with the NS group (right). Significantly reduced ratios were observed in the IS + True group compared with the sham tONS treatment ($n = 5$ rats/group, right). ## $P < 0.01$, IS + Sham vs. NS + Sham; * $P < 0.05$, ** $P < 0.01$, IS + True vs. IS + Sham; error bars represent SEM

amount of time spent resting or on unilateral grooming. These results showed that tONS could reduce the periorbital nociceptive thresholds and alleviate pain behaviors caused by IS stimulation in the adult rats, indicating that tONS has therapeutic effect in treating migraine-related pain, which consistent with our previous clinical research [21].

Activations of the TNC and PAG after IS-induced migraine condition

The c-Fos protein has always been recognized as a marker of neuronal activity, and the numbers of c-Fos-positive cells are essentially proportional to the intensity of neuronal stimulation [33]. Thus, we measured the differences in c-Fos expression induced by IS-or NS-modeled rats. Considering that occipital nerves as branches of C2 nerves involved in the information of TCC, which played important role in migraine regulation, the TNC (also known as Sp5C in animal atlas) was firstly chosen to be investigated [5, 6, 34, 35]. The results showed that the number of c-Fos positive cells in the IS group was significantly higher than that in the NS group in the TNC, and also increased in the area of the PAG, which further confirmed that the epidural stimulation of IS might activate the trigeminal neurovascular system pathway from the TNC to the PAG [36]. The stimulation of the PAG

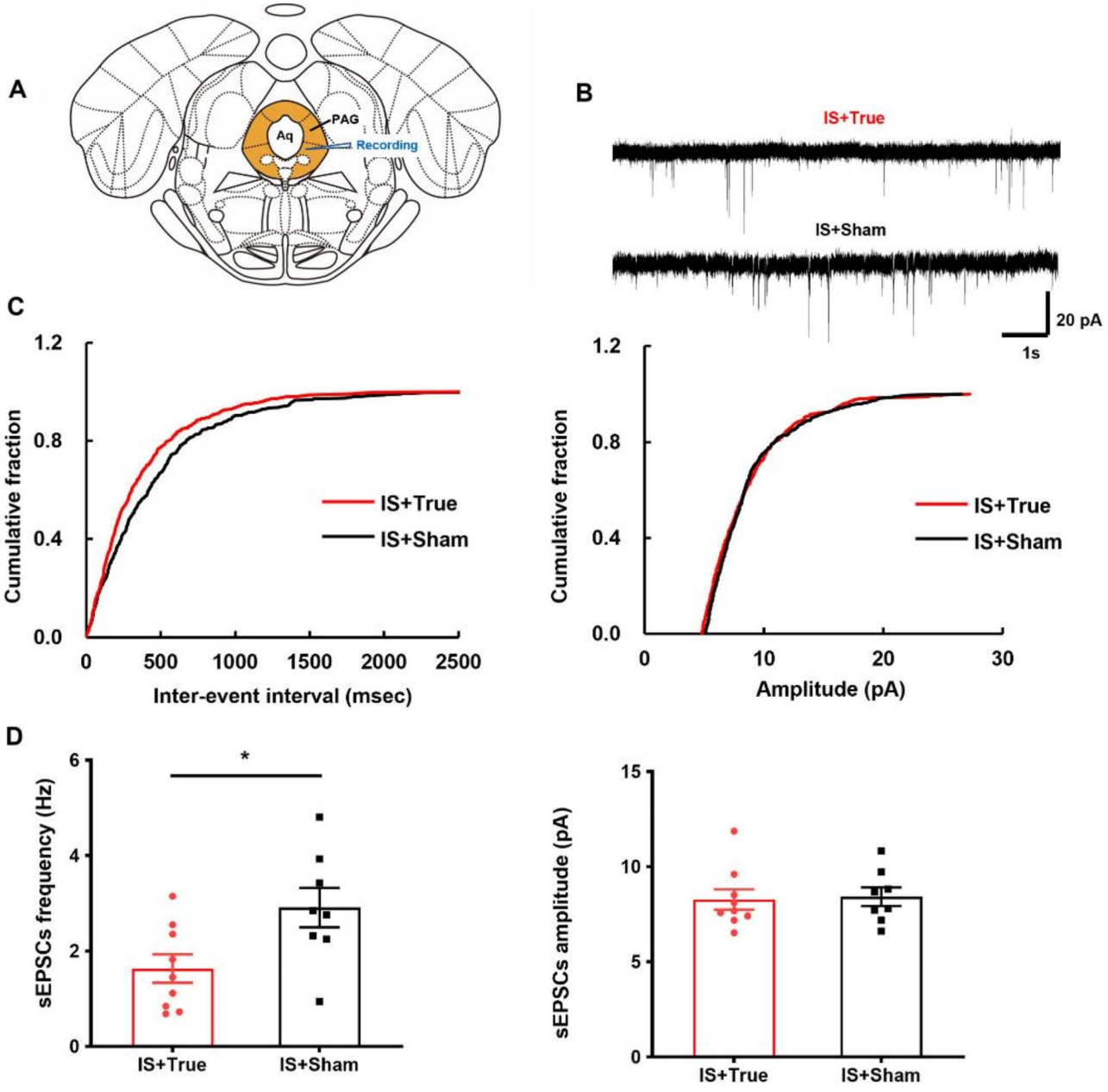

Fig. 7 tONS treatment attenuated synaptic transmission in the PAG of IS rats. (**A**) Schematic diagram showed the placement of recording electrodes in the PAG. (**B**) Representative traces of the sEPSCs recorded in the PAG neurons from true and sham tONS treatment of IS rats. (**C**) Cumulative fraction of inter-event interval (left) and amplitude (right) of the sEPSCs in the true and sham tONS treatment of IS rats. (**D**) Statistic results of the frequency (IS + True: $n = 9$ neurons/4 rats, IS + Sham: $n = 8$ neurons/4 rats; left) and amplitude (IS + True: $n = 9$ neurons/4 rats, IS + Sham: $n = 8$ neurons/4 rats; right) of sEPSCs. Significantly reduced frequency of sEPSCs were observed in the IS + True group compared with the sham tONS group. * $P < 0.05$, IS + True vs. IS + Sham; error bars represent SEM

has already been reported could provoke the onset of headache pain [37, 38]. Besides, the PAG activity can be modulated by various neuropeptides and neurotransmitters involved in migraine modulations, such as serotonin, orexin, and CGRP, suggesting the PAG as a possible site of action of headache treatments [38]. Our findings also highlighted the expressions of CGRP increased in the PAG after IS modeling, which supported its containing potential therapeutic targets for preventive treatment of migraine.

Possible mechanisms of tONS in regulating migraine

Our findings showed that the c-Fos expression significantly decreased in the IS groups of whom received true tONS treatments, with consistent levels in both the PAG and TNC, which indicated that tONS could reduce the

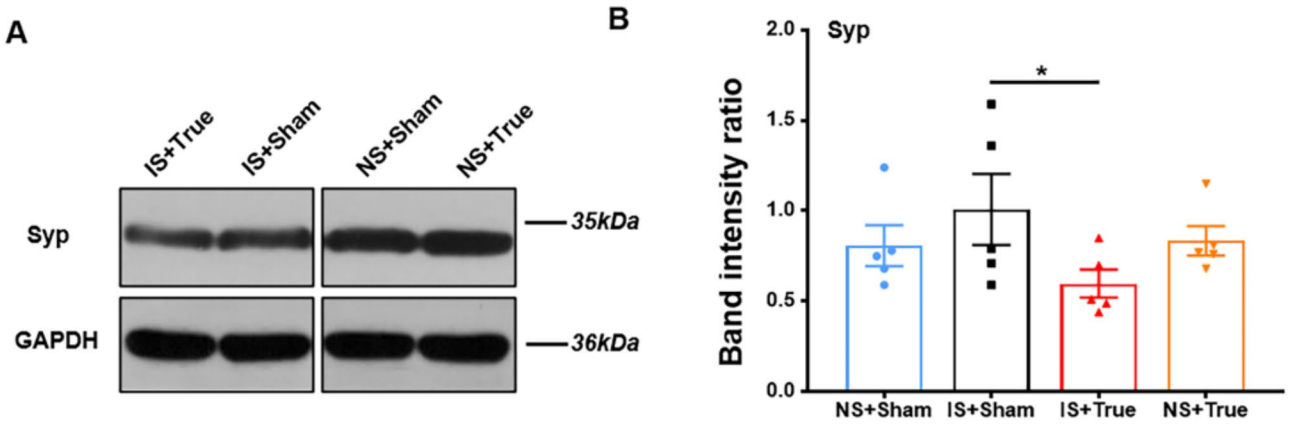

Fig. 8 Expression of Syp in the PAG alleviated after tONS treatment. (**A**) Typical western blot images of the Syp from the PAG in each of four groups. (**B**) The band intensity ratio of the Syp in the PAG decreased significantly after true tONS treatment compared with the sham tONS treatment groups ($n = 5$ rats/group, right). * $P < 0.05$, IS + True vs. IS + Sham; error bars represent SEM

neuronal activities in the TNC and PAG. Furthermore, studies have shown CGRP concentrations are elevated during migraine attacks and reduced during pain relief [39]. Vincent et al. found that immunologically detected CGRP activity in cervical venous blood was significantly reduced in rats stimulated by ONS relative to controls [40]. In the current study, the numbers of CGRP-positive cells in the PAG area among IS group were significantly higher than those in the NS group, indicating that IS administration led to higher localized CGRP expression in the PAG. After treatment with true tONS, CGRP expression in the IS group was significantly lower than that in the sham tONS group, suggesting that tONS treatment down-regulated CGRP expression in the PAG. These results were confirmed by western blotting experiment in the PAG region.

For further investigating the underlying mechanisms of tONS treatment, the whole-cell patch recording was performed in the PAG. We found that the frequency of sEPSCs was significantly attenuated in the IS + true group than in the IS + sham group, which indicated that tONS treatment could influence the excitability of the PAG in the rat migraine model. Due to the spontaneous events are thought to be the results of the presynaptic action potential evoked neurotransmitter vesicles release from the readily releasable pool [41], the Syp, a major integral membrane protein of presynaptic vesicles, had also been examined. The Syp is required for vesicle formation and exocytosis and is widely used as a marker for synaptic activity [42]. The data showed that the expression of Syp in the PAG decreased significantly after true tONS treatment compared with the sham tONS treatment, which supported the underlying mechanisms of tONS might related to the regulation of the excitability in the PAG, especially for presynaptic activity.

Clinical implications

Previous study demonstrated the clinical benefit of tONS in patients with migraine but could not rule out the placebo effect such as patients' expectation, due to limitations of clinical studies and could not investigate underlying mechanisms within patients [21]. Therefore, a similar apparatus was used for administering tONS in the current animal study and the results showed the significantly therapeutic effects of tONS in treating migraine. The mechanisms involved in the tONS indicated the synaptic plasticity of the PAG might play an important role in this nerve modulation, which provided clues for future optimization of treatment parameters and devices. Fully considering the results in the current studies and previous clinical trial, we could find that tONS as a non-invasive peripheral nerve electrical stimulation, is effective, safe and portable. It could be recommended as an alternative or complementary therapy for migraine prevention and might benefit patients who prefer nonpharmacological treatment.

Limitations

To the best of our knowledge, this is the first animal study assessed the effects of tONS and detected its possible underlying mechanisms. The present study also used multiple behavioral readouts and electrophysiological methods to strengthen the results. However, we should also notice the limitations of current study. As migraine is a female predominant disorder, it would be better using both female and male rats to more likely mimic clinical situation or at least taking female hormones in account. While, Maddahi et al. demonstrated that progesterone did not induce a significant change neither in basal level nor upon stimulated release of CGRP from dura mater or trigeminal ganglion in male or female rats when compared to the vehicle control [43]. Considering the present results had already shown that male rats had significant

differences among four groups, the female rats were not added in view of animal welfare and ethics. Furthermore, it is essential to investigate the precise synaptic mechanisms underlying tONS treatment for migraine using optogenetic or chemogenetic approaches. Although the current study provides preliminary evidence supporting the therapeutic effects and potential mechanisms of tONS in adult male rats, further research is needed to elucidate the detailed synaptic mechanisms and extend the findings to multicenter clinical studies.

Conclusions

Taken together, the present study demonstrated the tONS could alleviate migraine related pain by stimulating the bilateral occipital nerves, affecting TCC function and altering c-Fos and CGRP expression in the trigeminal neurovascular system, especially in the TNC and PAG. tONS may play a therapeutic role by modulating synaptic plasticity in the PAG of adult migraine rats. It could be recommended as an alternative or complementary therapy for migraine prevention, and it is essential to determine the precise mechanisms involved in the further studies.

Abbreviations

ACSF	Artificial cerebrospinal fluid
CGRP	Calcitonin gene-related peptide
DAPI	4′,6-diamidino-2-phenylindole
ENS	Electric neurostimulation
IS	Inflammatory soup
NS	Normal saline
ONS	Occipital nerve stimulation
PAG	Periaqueductal gray
PBS	Phosphate buffered saline
PLA	People's Liberation Army
PTX	Picrotoxin
SEM	Standard error of the mean
sEPSCs	Spontaneous excitatory postsynaptic currents
Syp	Synaptophysin
TENS	Transcutaneous electrical neurostimulation
tONS	Transcutaneous occipital nerve stimulation
TCC	Trigeminal cervical complex
TNC	Trigeminal nucleus caudalis
YLDs	Years lived with disability

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Author contributions
Conceptualization: YLL, SYY, XH; Methodology and investigation: YLL performed most of the behavior and western blot experiments. SLG performed most of immunofluorescence studies. YLL and YL were responsible for electrophysiological experiments. YL and JRM performed some of behavior and biochemical studies. YLL, XXL and RZL pooled the data for analysis. RZL and DFZ were responsible for optimizing the pictures; Funding acquisition: YLL, XH and RZL; Supervision: XH, SYY; Writing– original draft: YLL, SLG, XH; Writing– review & editing: YLL, DZ, SYY, HX. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal experiments performed in this study were approved by the Committee on Animal Use for Research and Education of the Laboratory Animals Centre at Chinese PLA General Hospital.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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