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ORIGINAL RESEARCH

Muscarinic Receptor-Mediated Electroacupuncture Modulation of Reactive Enteric Glial Cells Ameliorates Postoperative Ileus

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Background: Emerging evidence highlights reactive enteric glial cells (EGCs) as pivotal players in the pathogenesis of postoperative ileus (POI). Recent studies have demonstrated that electroacupuncture (EA) Zusanli acupoint (ST 36) effectively alleviates POI. This study aims to investigate the underlying mechanisms of EA ST 36-mediated functional restoration of POI.

Methods: A standardized intestinal manipulation (IM) procedure was conducted to establish the POI murine model. Mice were then treated with EA ST 36, and gastrointestinal (GI) motility function was measured after 24 hours. Immunofluorescence, Western blot, and hematoxylin-eosin staining were used to assess intestinal inflammation, the expression of glial fibrillary acidic protein (GFAP) and type 3 muscarinic acetylcholine receptors (m3AChRs). Vagus nerve activity was evaluated through plasma enzyme-linked immunosorbent assay combined with heart rate variability.

Results: Compared to the Sham group, animals in the IM group demonstrated GI dysfunction characterized by delayed whole gut transit, prolonged colonic bead expulsion time, and reduced heart rate variability, which accompanied by decreased acetylcholine levels, elevated Chiu's scores, upregulated GFAP expression, and downregulated m3AChRs expression. EA ST 36 effectively mitigated these changes and enhanced c-Fos protein expression in the nucleus tractus solitarius. Fluorocitrate (a glial cell inhibitor) and carbachol (a cholinergic agonist) replicated the effects of EA ST 36, which was abolished by either pretreatment with J104129 (a specific m3AChR antagonist) or surgical vagotomy.

Conclusion: The results suggest that EA ST 36 exerts its effects through vagally-mediated modulation of m3AChR signaling, which involves the reduction of the reactivity of EGCs and the improvement of GI dysfunction induced by IM.

Keywords: electroacupuncture, postoperative ileus, enteric glial cells, type 3 muscarinic acetylcholine receptors

Introduction

Postoperative ileus (POI) represents a common gastrointestinal (GI) complication following abdominal surgery, characterized by impairment of GI motility.[1,2] The pathogenesis and etiological factors of POI following abdominal surgery have not yet been fully elucidated. This condition may involve factors such as intraoperative intestinal surgical manipulation, perioperative use of opioid analgesics, and postoperative stress response.[3] These factors collectively lead to neuroregulatory disorders and inflammatory responses, thereby contributing to the development of POI.[4] Clinically, POI exhibits high incidence rates and is associated with prolonged hospitalization, increased healthcare costs, and elevated risks of postoperative complications.[5,6] These clinical consequences underscore the need for better understanding of its underlying mechanisms.

The GI motility regulation system comprises an intricate network integrating enteric neurons, enteric glial cells (EGCs), smooth muscle cells, and intestinal cells of Cajal (ICCs). This enteric neuron-ICC-smooth muscle cellular complex coordinates physiological and pathophysiological processes through neuromuscular interactions.[7] Emerging evidence

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highlights EGCs as critical modulators in motility disorders, particularly through their transition into reactive states.[8,9] EGCs serve as a pivotal hub in maintaining GI motility homeostasis through dynamic regulation of enteric neurotransmitter balance and neuroglial network communication, with their functional dysregulation directly leading to neuromuscular signaling abnormalities.[10] Following noxious stimulus exposure, EGCs undergo phenotypic activation into reactive states through a complex series of pathways that affect the enteric nervous system (ENS) and immune cell properties, which ultimately affects GI motility.[11] Both quantitative and functional abnormalities in EGC populations can induce intestinal dysmotility, manifesting as delayed emptying and impaired luminal transport.[12]

Vagal efferent fibers establish direct synaptic connections with nearly all postganglionic neurons in the gastric and proximal duodenal plexus,[13] forming excitatory acetylcholine (ACh) and inhibitory non-adrenergic and non-cholinergic pathways that influence GI motility.[14] Stimulation of the vagus nerve, through cholinergic anti-inflammatory pathways and/or its afferent nerves, may reduce inflammation levels by activating the hypothalamic-pituitary-adrenal axis.[15,16] Notably, functional neuro-glial interactions occur at myenteric plexuses where cholinergic neurons release neurotransmitters (particularly ACh and ATP) to activate EGCs.[17,18] EGCs express a multitude of receptors in response to their direct and indirect activation and inhibition by various transmitters in the ENS.[11] Recently, type 3 muscarinic ACh receptors (m3AChRs) have been identified on the surface of EGCs,[17] which can respond to a range of transmitter signals from the ENS. Moreover, the regulation of this receptor enables the modulation of GI motility.[17] Therefore, the vagus nerve precisely regulates GI function and inflammatory levels through multiple pathways and multicellular interactions. However, surgical transection of the vagus nerve can affect normal physiological functions to a certain extent, such as leading to reduced food and water intake, an increased risk of inflammatory bowel disease, and even a higher risk of mortality.[19,20]

Mounting evidences support the safety and efficacy of carrying out acupuncture perioperatively.[21–25] Studies have demonstrated that electroacupuncture at Zusanli (EA ST 36) activates the vagal-adrenal anti-inflammatory axis through the dorsal root ganglion, effectively ameliorating POI.[26,27] EA ST 36 can also improve the deformation of EGCs caused by blood loss through the vagus nerve.[28] Compared with invasive vagus nerve stimulation requiring surgical electrode implantation, EA ST 36 represents a minimally invasive therapeutic alternative that eliminates procedural trauma while maintaining neuromodulatory efficacy.[29,30] Despite the recognized reactivity of EGCs in GI pathophysiology, the downstream effector and precise regulatory mechanisms of activated EGCs in POI remain largely unexplored, particularly regarding their interplay with extrinsic neural circuits. In the present study, we investigated the vagally mediated pathway through which EA ST 36 ameliorates POI-related dysmotility, with specific emphasis on the cholinergic anti-inflammatory axis.

Materials and Methods

Mice

Male C57BL/6J mice (Rise Mice Biotechnology Co., Ltd., Guangdong, China) were 6–8 weeks old, 20–25 g body weight, and housed under specific pathogen free conditions. The animal facility maintained a controlled environment with: 12/12-hour light/dark cycles, ambient temperature of 22–26°C, and relative humidity of 40–60%. All animals received ad libitum access to standardized rodent chow and drinking water throughout the experimental period. Randomization protocols were implemented during both group assignment and experimental procedures.

Establishment of POI Model

POI was surgically induced through standardized intestinal manipulation (IM) as previously described.[31] Mice were anesthetized using isoflurane, followed by aseptic preparation of the abdominal region. A midline laparotomy was performed through sequential incision of the skin and abdominal muscle layers. The small intestine was gently exteriorized and placed on saline-moistened sterile gauze. Two sterile cotton swabs were used to apply controlled mechanical stimulation by stroking the intestinal serosa along its longitudinal axis (2–3 times, lasted 5 minutes). Following manipulation, the bowel was gently repositioned and the abdominal wall was reconstructed in layers using interrupted 4–0 sutures. Animals were monitored until regaining righting reflex. Sham group underwent identical surgical procedures excluding the IM protocol.

EA ST 36

Mice were anesthetized and loosely immobilized for EA procedures. Bilateral ST 36 (2 mm lateral to the tibial tuberosity, 4 mm distal to the knee joint) received 3-mm-deep acupuncture needle insertions. Electrical stimulation (HANS-200E, China, 1 mA, 10 Hz, 0.4 ms)[32] was delivered for 30 minutes post-needling. Mice in IM+ShamEA group underwent identical needle placement without electrical stimulation.

Selective Neurectomies

Selective neurectomy was performed 7 days prior to EA. Left cervical vagotomy (LCV): Mice were anesthetized and the cervical region was aseptically prepared. A midline incision exposed the sternocleidomastoid muscle, which was retracted to visualize the carotid sheath. Target nerves were ligated proximally and distally with 4–0 nylon sutures prior to mid-segment transection.[33] Sub-diaphragmatic vagotomy (SDV): Following abdominal depilation and antiseptic preparation, a midline laparotomy was performed. The gastroesophageal junction was microsurgically exposed, allowing identification of the vagus nerve's ventrodorsal trunks. 5-mm sub-diaphragmatic nerve segments were bilaterally excised using microscissors.[32]

Drugs Administration

Referring to Sun et al,[34] fluorocitrate (FC, a glial cell inhibitor) (Sigma, F9634) was prepared as a 1 μmol/mL FC solution and administered intraperitoneally at 20 μmol/kg daily for 7 days. Carbachol (Macklin, C838452-1g, a cholinergic agonist) and J104129 fumarate (MCE, 257603–40-0, a specific m3AChR antagonist) were prepared as a -0.0125 mg/mL solution in 0.9% NaCl, with a dosage of 0.2 mg/kg for each mouse.[35]

GI Motility Evaluation

Postoperative GI motility was quantified by assessing the distribution of FITC-dextran in the intestine after 24 hours.[36] FITC-dextran (Sigma, 46945–100 mg-F) was prepared as a 50 mg/mL solution in 0.9% NaCl and administered via oral gavage (0.1 mL/mouse) 22.5 hours post-surgery. Following a 90-minute interval, mice were euthanized through anesthetic overdose. The intestinal tract was systematically divided into 15 segments: segment 1 (stomach), segments 2–11 (equally divided small intestine), segment 12 (cecum), and segments 13–15 (equally divided colon). Intestinal contents were collected in PBS-containing centrifuge tubes (1 mL PBS), centrifuged at 12,000 rpm for 15 minutes using a pre-chilled high-speed centrifuge, and supernatants stored protected from light at 4°C. Fluorescence intensity was quantified using a microplate reader (Thermo Fisher Scientific, USA, excitation 494 nm/emission 521 nm). GI transit was assessed through the geometric center (GC) calculation: $GC = \Sigma$ (% total fluorescence per segment × segment number)/100.

Food Intake

Mice were placed in clear cages with ad libitum access to water at 3 hours post-operation. Food consumption was quantitatively measured after an additional 3-hour period.

Colonic Bead Expulsion Time

Six hours postoperatively, mice were anesthetized with isoflurane, and a 2-mm bead was quickly and gently placed in the anus of the mice using serrated forceps. A $6^{\#}$ gavage catheter was used to gently push the bead into the proximal colon 2 cm away from the anus.[37]

Heart Rate Variability (HRV)

After anesthetizing the mice, and their limbs were loosely fixed to a foam plate. Four diminutive needles were inserted into the limbs and attached to recording electrode clips for recording for no less than 10 minutes.

Hematoxylin-Eosin (HE) Staining

Small intestine tissues were paraffin-embedded, dewaxed, rehydrated, and then HE stained. The sections were then scored using Chiu's intestinal injury histological score by a non-in-house researcher. The Chiu's scoring system as follows: Grade 0: Intact villous architecture with preserved epithelial integrity; Grade 1: Intermittent widening under the tip of villus; Grade 2: Villi apical epithelium exfoliation and ulceration; Grade 3: Villi apex destruction extended to the base; Grade 4: Complete epithelial loss; Grade 5: Lamina propria collapse with ulceration and bleeding.[38]

Enzyme Linked Immunosorbent Assay (ELISA)

Plasma ACh content analysis was performed according to the company's protocol (Cat No. MM-0520M2, MEIMIAN).

Immunofluorescence (IF) Staining

The longitudinal muscle and myenteric plexuses and brain sections were blocked with a rapid blocking solution (Cat No. P0260, Beyotime) and incubated with the primary antibodies against glial fibrillary acidic protein (GFAP), c-Fos, and m3AChR (as shown in Table 1). After a three-time washing procedure, the samples were incubated with the secondary antibodies (Table 1). The samples were washed three times. Subsequently, the samples were sealed with DAPI-containing anti-fluorescence quencher (Cat No. P0131-25 mL, Beyotime). Fluorescence images were taken using an automatic confocal microscope (Nikon, Japan) with identical parameters for all samples. Three fields or sections of each sample were randomly selected under the microscope and imaged, and the mean value was calculated after analysis by Image J software.

Western Blot (WB)

The isolated jejunal tissues were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using standard WB techniques. The membranes were blocked with rapid blocking solution (Cat No. P30500, NCM Biotech) and incubated with the primary antibodies for GFAP and GAPDH (Table 1). After washing off the excess primary antibody, the IgG HRP secondary antibody was incubated for one hour at room temperature. After washing off the excess antibody, the bands were photographed by applying the luminescent solution. The experiment was conducted in triplicate for each sample.

Statistical Analysis

All data analysis was conducted using the statistical software packages SPSS 25.0 software (SPSS Inc., Chicago, USA) and GraphPad Prism 8 (GraphPad Prism Software Inc., San Diego, USA). The data are presented as the mean ± standard deviation. The assumption of normality was tested using the Shapiro–Wilk test. A two-sample *t*-test was employed for two independent samples with homogeneity of variance. One-way ANOVA was used to compare the quantitative data between multiple groups in accordance with normal distribution. LSD was used for post-hoc test of components if the variance was homogeneous. Tamham's T2 was used for post-hoc test of components if the variance was not homogeneous. The non-parametric test was used to compare the quantitative data between multiple groups that did not meet the normal distribution, and the non-parametric test Bonferroni correction was used for the post-hoc tests. $P<0.05$ was considered statistically significant.

Table 1 Information About Primary and Secondary Antibodies Used

Antibody	Host Species	Dilution	Catalog Number	Source
Anti-GFAP antibody	Chicken	1: 500	ab4674	Abcam (Waltham, MA, USA)
	Rabbit	1: 10,000	16,825-1-AP	Proteintech (Wuhan, China)
Anti-c-Fos antibody	Rabbit	1: 500	2250 S	Cell Signaling Technology (Danvers, MA, USA)
Anti-CHARM3 antibody	Rabbit	1: 200	AMR-006	Alomone Labs (Jerusalem, Israel)
Anti-GAPDH antibody	Rabbit	1: 15,000	16,825-1-AP	Proteintech (Wuhan, China)
Horseradish preoxidase-conjugated anti-rabbit antibody	Goat	1: 10000	Ab205718	Abcam (Waltham, MA, USA)
Alexa Fluor® 488- conjugated anti-chicken antibody	Goat	1: 500	ab150169	Abcam (Waltham, MA, USA)
Alexa Fluor® 488- conjugated anti-rabbit antibody	Goat	1: 500	ab150077	Abcam (Waltham, MA, USA)
Alexa Fluor® 594- conjugated anti-rabbit antibody	Donkey	1: 500	A-21207	Thermo Fisher scientific (Waltham, MA, USA)

Results

EA ST 36 Improves Postoperative GI Dysfunction and Intestinal Injury, with m3AChRs Expressed on EGCs

We replicated the POI model by IM to investigate the impact of EA ST 36 on GI motility (Figure 1A and B). In the Sham group, FITC moved fast, and the peak GC of fluorescence signal appeared in the 9–10 segments of the small intestine at 90 minutes (Figure 1C), and the colonic bead was expelled in about 1 minute (Figure 1D). In the IM group, FITC-dextran appeared in the 3–4 segments after IM, which was significantly slower than that in the Sham group (P=0.000), and the colonic bead expulsion time was significantly longer than that in the Sham group (P=0.009). There was a meaningful increase in perioperative weight loss (P=0.004, Figure 1E), and food intake was diminished (P=0.000, Figure 1F); Similarly, the intestinal inflammation scores (Chiu's score) were significantly increased in comparison to the Sham group (P=0.000, Figure 1G and H). There was no statistically significant difference between the IM+ShamEA group and the IM group in GC (P=0.544), colonic bead expulsion time (P=0.275), perioperative weight loss (P=0.133), food intake (P=0.801) and Chiu's score (P=0.996). Compared with the IM group and the IM+EA group, EA significantly improved

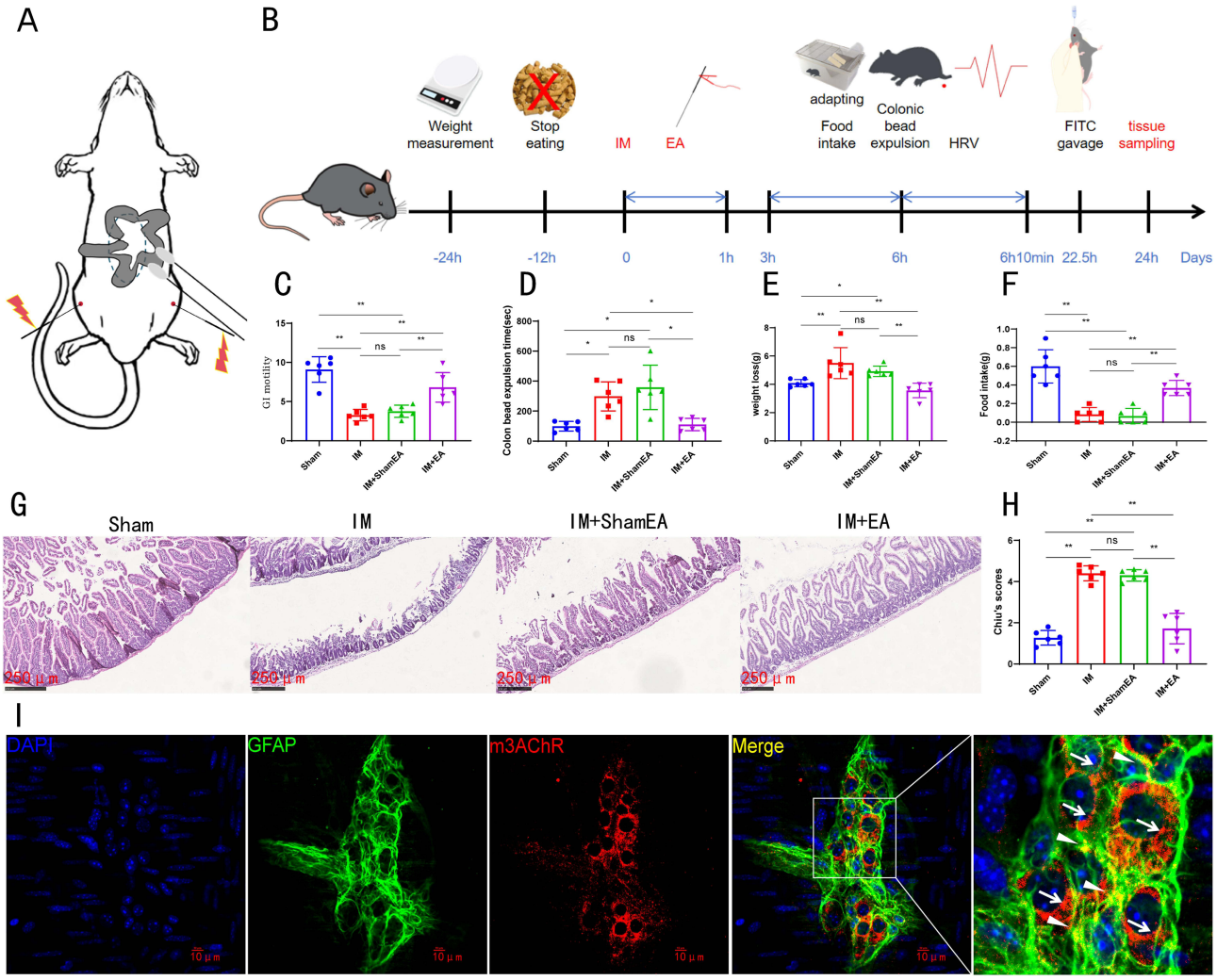

Figure 1 EA alleviated IM-induced GI motility dysfunction and inflammation of small intestinal muscle layer. (**A**) IM modeling and EA ST 36 intervention diagram. (**B**) Schematic representation of the experimental protocol. (**C**) The mean GI transit at the 24th hour after surgery (n=6). (**D**) Colonic bead expulsion time at the 6th hour after surgery (n=6). (**E**) Perioperative weight loss (n=6). (**F**) Food intake from the 3rd hour to the 6th hour after surgery (n=6). (**G** and **H**) HE staining of small intestine and Chiu's scores (n=6). (**I**) IF staining of GFAP and m3AChRs in intestinal myenteric plexus (The white arrow indicates the co-localization of m3AChRs and enteric neurons, and the white triangles indicates immunofluorescently co-localization of GFAP and m3AChRs). One-way ANOVA test with post-hoc test *P < 0.05, **P < 0.01, P>0.05.
Abbreviations: ns, not significant.

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GI peristalsis (GC P=0.001, colonic bead expulsion time P=0.011), decreased weight loss (P=0.000), food intake (P=0.001), and Chiu's score (P=0.000).

Subsequently, to further investigate the mechanism of EA associated with EGCs, we then performed IF staining of EGCs and m3AChRs (Figure 1I). As shown in the figure, there was IF co-labeling of m3AChRs with enteric neurons (indicated by white arrows). Notably, the EGCs marker GFAP was partially immunofluorescently co-labeled with m3AChRs (indicated by white triangles). This suggests that EGCs and/or enteric neurons may express m3AChRs and may be potential targets for EA-mediated improvement of GI motility.

EA ST 36 Activates Vagus Nerve and Reduces GFAP Expression, Potentially Involving m3AChRs

In postoperative GI dysfunction, vagal-sympathetic balance changes, vagus nerve and sympathetic nerve excitability may be changed, and vagus nerve is inhibited.[4,39] To assess changes in vagal tone, HRV was measured. IM group, compared with the Sham group, showed decreases in HRV: RR interval was shortened (P=0.000, Figure 2A), LF/HF was increased (P=0.026, Figure 2B), and HFP was decreased (P=0.004, Figure 2C). However, after ShamEA treatment, the above indicators did not obviously change and the attenuated HRV did not improve (RR interval, P=0.731; LF/HF, P=0.958; HFP, P=0.892). Treatment with EA ST 36 resulted in a significant improvement in HRV (RR interval, P=0.002; LF/HF, P=0.019; HFP, P=0.034). Subsequently, we tested the changes in plasma ACh levels by ELISA, we found that the amount of ACh was reduced in IM-induced POI mice (P=0.000, Figure 2D), but plasma ACh was increased to a certain extent after EA treatment, and this difference was found to be statistically significant (P=0.000). ShamEA treatment did not seem to have an effect (P=0.329). In order to further understand the central mechanism of EA regulating POI, we performed IF analysis of the nucleus tractus solitarius (NTS) of the vagal afferents in the mouse brain (white dotted lines in Figure 2E). As shown in Figure 2F, comparing with the Control group, c-Fos protein amount in the NTS of the EA group was significantly increased (P=0.005), indicating that EA is associated with activation of the vagus nerve center. In addition, IF staining results of the intestinal muscle layer showed that the EGCs changed to reactive EGCs after IM (P=0.000, Figure 2G and H), and the expression of m3AChRs decreased (P=0.000, Figure 2G–I), but no difference was found after shamEA treatment (GFAP, P=0.664; m3AChRs, P=0.52). However, after EA ST 36, the reactive EGCs were reduced (P=0.000), and the expression of m3AChRs was also significantly increased (P=0.001). Additionally, the assessment of GFAP expression through WB analysis yielded comparable outcomes (Figure 2J and K).

Reactive EGCs Implicate in Development of GI Dysfunction in POI Mice

FC is a selective inhibitor of glial cell function. It inhibits glial cell function without causing intestinal inflammation by inhibiting cisaconitase in the tricarboxylic acid cycle and interfering with energy metabolism.[40] To explore the potential role of reactive EGCs in the pathogenesis of POI, we administered FC intraperitoneally to mice for 7 consecutive days to specifically inhibit EGCs from converting to reactive EGCs phenotype (Figure 3A). By measuring GI transport function at the 24th postoperative hour, we found that compared with the NS+IM group, the POI mice given FC had improved GI transport function (P = 0.014, Figure 3B), colonic bead expulsion time (P =0.029, Figure 3C), and perioperative weight loss (P=0.006, Figure 3D) and food intake at the 3rd hour after surgery (P=0.001, Figure 3E) were also significantly increased. In addition, during the one-week administration period, there was no significant difference in body weight change between both groups (Figure 3F), and the hair, behavior and eating of the mice were as normal, indicating that FC did not cause toxic effects in the mice. IF results at the 24th hour after surgery showed that the EGCs were significantly inhibited and the transition into reactive EGCs was reduced after the administration of FC (P=0.000, Figure 3G and H), and the results were also supported by WB (P=0.045, Figure 3I and J). This suggests that inhibiting the transition of EGCs to reactive EGCs can promote the recovery of GI function after surgery.

M3AChRs Mediate the Improvement of GI Dysfunction in POI Mice by EA

The above experiments found that m3AChRs played a crucial role in EA improving postoperative GI dysfunction. Therefore, to more deeply explore the role of m3AChRs in EA improving postoperative GI dysfunction, we used AChR agonist carbachol and specific m3AChRs antagonist J104129 (Figure 4A). After administration of carbachol, the GI

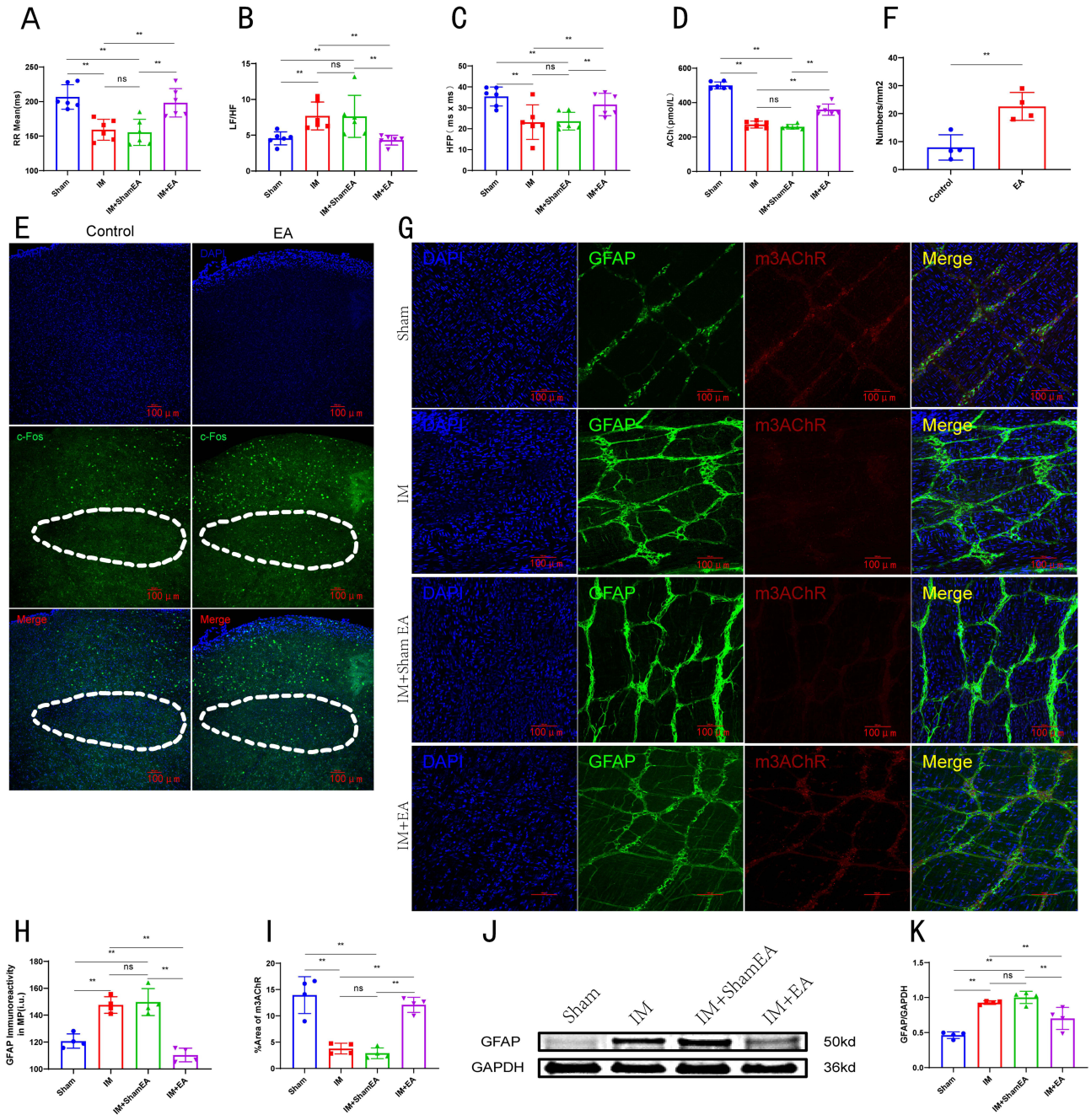

Figure 2 EA ST 36 activates the vagus nerve and increases m3AChRs expression while reducing GFAP expression. Changes of HRV at 6 hours after surgery: RR mean (**A**), LF/HF (**B**), and HFP (**C**) (n=6). (**D**) Plasma ACh expression by ELISA (n=6). (**E** and **F**) Changes of c-Fos protein amount in NTS by IF staining (n=4) (NTS in the mouse brain is shown by the white dotted lines). (**G-I**) Changes of GFAP and m3AChRs expression in intestinal myenteric plexus by IF staining (n=4). (**J** and **K**) Changes of GFAP expression in the small intestine by WB (n=4). One-way ANOVA test with post-hoc test, ** $P < 0.01$, $P > 0.05$.
Abbreviation: ns, not significant.

transport function of POI mice was improved (P=0.000, Figure 4B) and the colonic bead expulsion time was significantly reduced (P=0.000, Figure 4C) as compared with NS+IM group. IF staining results showed that GFAP was decreased and the transition from EGCs to reactive EGCs was reduced after carbachol administration (P=0.000, Figure 4D and E), and WB bands showed the similar results (P=0.003, Figure 4F and G). We subsequently administered J104129 before EA treatment. Compared with NS, the therapeutic effect of EA in POI mice was abolished after J104129 administration (P=0.002, Figure 4H; P=0.000, Figure 4I). IF staining and WB also showed that the GFAP increased after J104129

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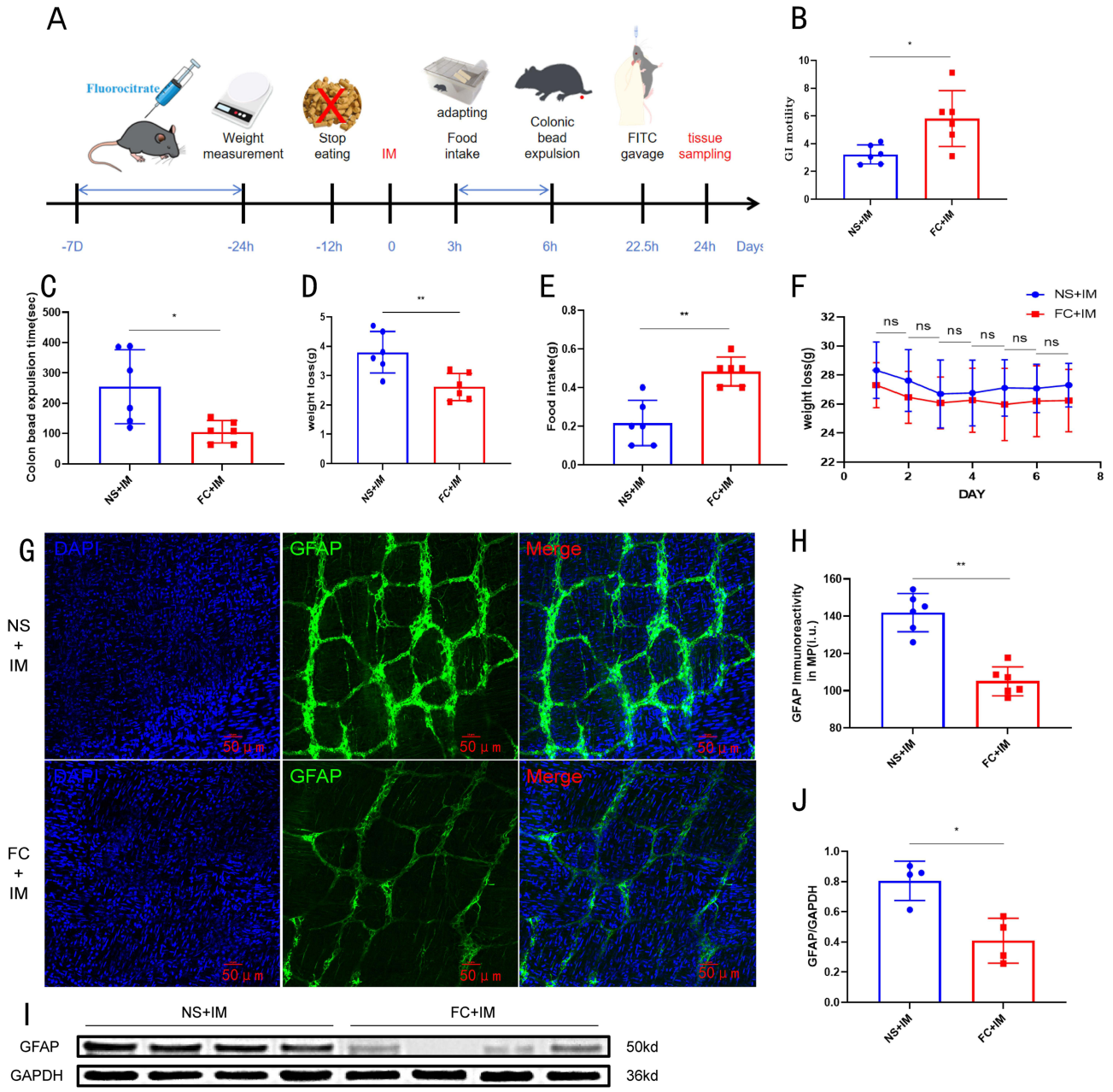

Figure 3 FC inhibited reactive EGCs and improved GI dysfunction in POI mice. (**A**) Schematic representation of the experimental protocol. (**B**) The mean GI transit at the 24th hour after surgery (n=6). (**C**) Colonic bead expulsion time at the 6th hour after surgery (n=6). (**D**) Perioperative weight loss (n=6). (**E**) Food intake from the 3rd hour to 6th hour after surgery (n=6). (**F**) Weight changes during intraperitoneal administration (n=6). (**G** and **H**) Changes of GFAP expression in intestinal myenteric plexus by IF staining (n=6). (**I** and **J**) Changes of GFAP expression in the small intestine by WB (n=4). A two-sample *t*-test * $P < 0.05$, ** $P < 0.01$, $P > 0.05$.
Abbreviation: ns, not significant.

administration (P=0.000, Figure 4J and K; P=0.030, Figure 4L and M). These results suggest that m3AChRs may play an essential role between reactive EGCs and EA in the treatment of POI.

Vagotomy Abolishes the Protective Effect of EA ST 36

In the regulation of GI motility, the vagus nerve plays an irreplaceable role. For a better understanding of the influence of the vagus nerve in EA, we performed LCV (Figure 5A) and SDV (Figure 6A) in normal C57 mice. Then, POI was induced by IM and EA ST 36 was given.

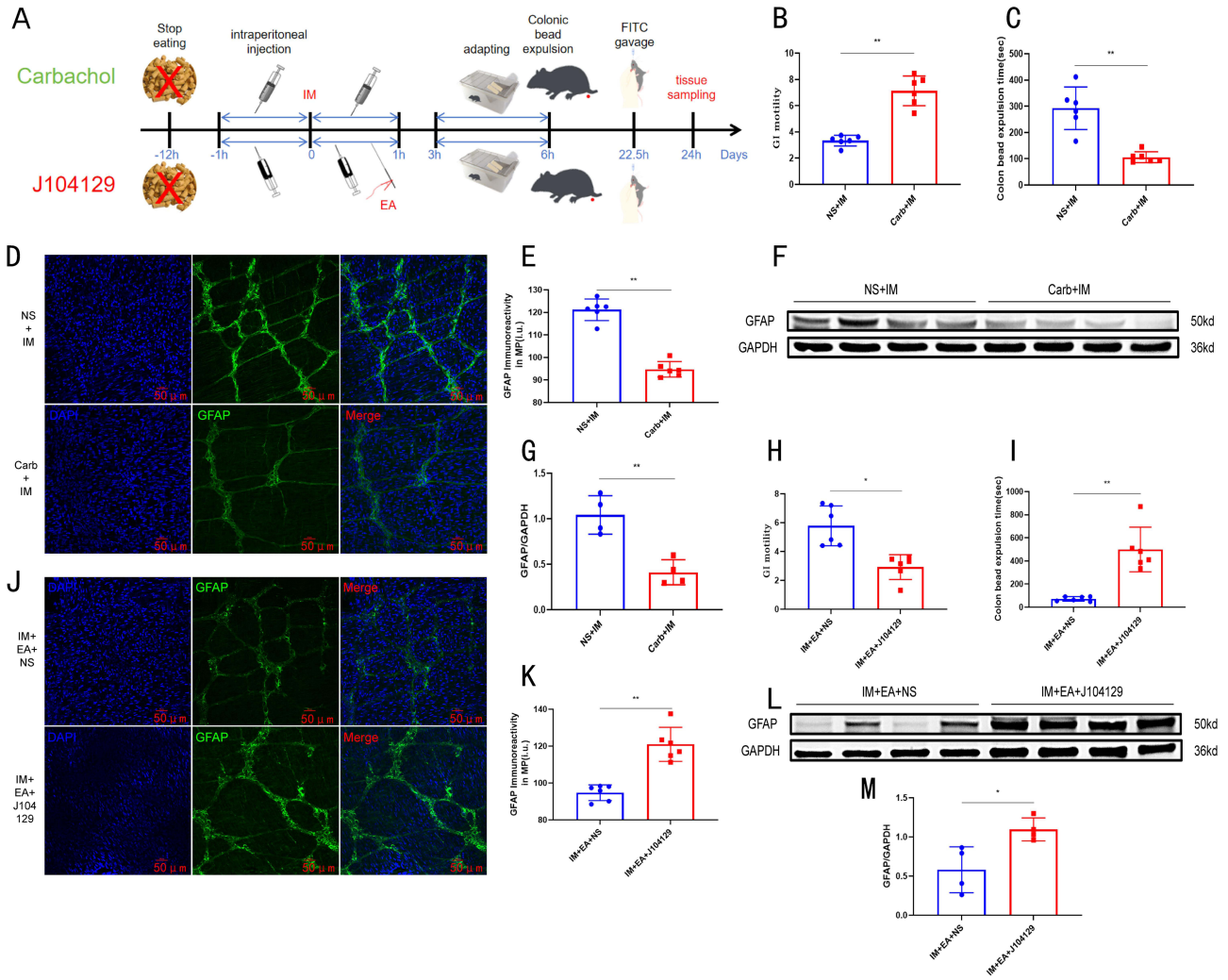

Figure 4 M3AChRs were involved in improving postoperative GI dysfunction in POI mice. Carbachol -AChRs agonist, J104129-m3AChRs antagonist. (**A**) Schematic representation of the experimental protocol. (**B–H**) The mean GI transit at the 24th hour after surgery (n=6). (**C–I**) Colonic bead expulsion time at the 6th hour after surgery (n=6). (**D, E** and **J, K**) Changes of GFAP expression in intestinal myenteric plexus by IF staining (n=6). (**F–G** and **L–M**) Changes of GFAP expression in the small intestine by WB (n=4). A two-sample *t*-test * $P < 0.05$, ** $P < 0.01$, $P > 0.05$.
Abbreviation: ns, not significant.

After IM-induced POI in LCV-pretreated mice, the GI transit function was decreased (P=0.001, Figure 5B), the colonic bead expulsion time was longer (P=0.000, Figure 5C), food intake was decreased (P=0.046, Figure 5D), Chiu's scores underwent an increase (P=0.000, Figure 5E and F), and GFAP expression increased (P=0.000, Figure 5G and H; P=0.004, Figure 5I and J). Mice pretreated with SDV also showed the same GI motility dysfunction after IM (P=0.000, Figure 6B; P=0.012, Figure 6C; P=0.007, Figure 6D; P=0.000, Figure 6E and F; P=0.000, Figure 6G and H; P=0.000, Figure 6I and J). However, after EA ST 36 treatment, the whole gut transit (LCV, P=0.436; SDV, P=0.885), colonic bead expulsion time (LCV, P=0.856; SDV, P=1.000) and food intake (LCV, P=0.960; SDV, P=0.685) did not change significantly. In addition, no treatment effect of EA ST 36 was observed on HE staining of the small intestine (LCV, P=0.411; SDV, P=0.135). GFAP in the small intestinal myenteric plexus was determined by IF staining (LCV, P=0.659; SDV, P=0.890) and WB (LCV, P=0.438; SDV, P=0.148), which showed that vagotomy in advance eliminated the therapeutic effect of EA ST 36 including the improvement of GI dysfunction on POI mice. Taken together, these results provide sufficient reason to believe that the vagus nerve is an irreplaceable key link in the treatment of POI by EA.

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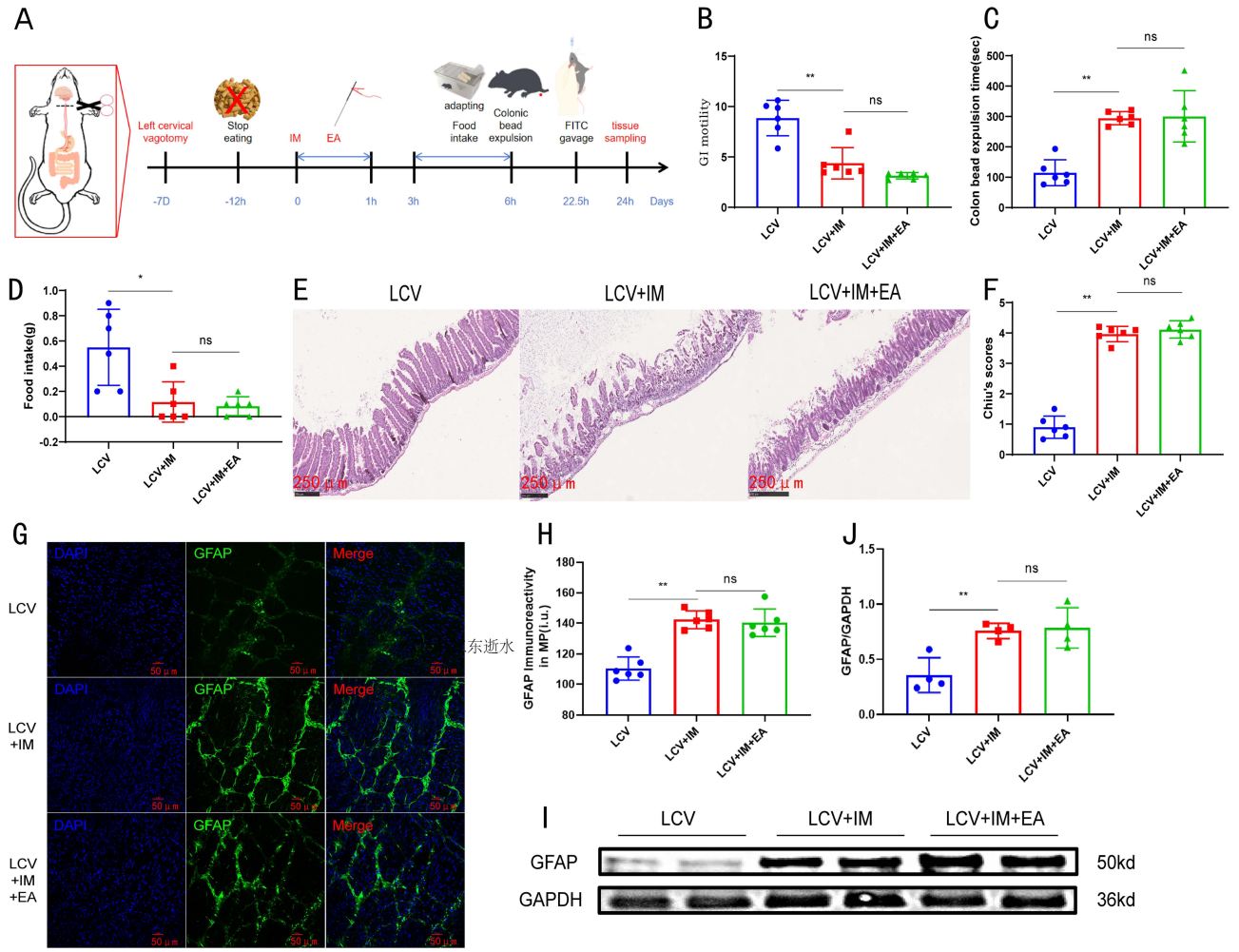

Figure 5 Loss of EA protection after LCV. (**A**) Schematic representation of the experimental protocol. (**B**) The mean GI transit at the 24th hour after surgery (n=6). (**C**) Colonic bead expulsion time at the 6th hour after surgery (n=6). (**D**) Food intake from the 3rd hour to the 6th hour after surgery (n=6). (**E** and **F**) HE staining and Chiu's score for small intestine (n=6). (**G** and **H**) Changes of GFAP expression in intestinal myenteric plexus by IF staining (n=6). (**I** and **J**) Changes of GFAP expression in the small intestine by WB (n=4). One-way ANOVA test with post-hoc test * $P < 0.05$, ** $P < 0.01$, $P > 0.05$.
Abbreviation: ns, not significant.

Discussion

Identification of EGCs as critical regulators in POI pathogenesis may provide a promising therapeutic target and novel research direction for the development of preventive and therapeutic strategies against POI.[34] In this study, we explored how EA ST 36 ameliorates POI through vagus nerve-mediated regulation of EGCs. Our findings demonstrate that EA ST 36 significantly enhanced GI transit recovery, which involved the increased plasma ACh levels, activated vagus nerve-m3AChRs signaling, and suppressed IM-induced EGCs hyperactivation. These results are consistent with clinical observation showing EA ST 36 improves GI motility and attenuates intestinal inflammation in POI patients.[41]

By measuring postoperative whole gut transit and colonic bead expulsion time post-surgery, we evaluated the recovery of GI motility in POI mice. In the current study, EA ST 36 significantly alleviated IM-induced delay in whole gut transit and prolonged colonic bead expulsion time. This finding is consistent with the results of animal experiments and clinical observations conducted by Liu and his team.[23,27] Quantitative assessment food intake as a key metabolic parameter revealed that EA ST 36 effectively prevented IM-induced decline in food intake. The underlying mechanism may involve multiple synergistic pathways. First, EA ST 36 promotes the secretion of plasma gastrin and motilin,[42] with the former enhancing digestive function by stimulating gastric acid secretion and mucosal repair, and the latter accelerating gastric sinus contractions to improve gastric emptying efficiency.[43] Second, EA ST 36 inhibits

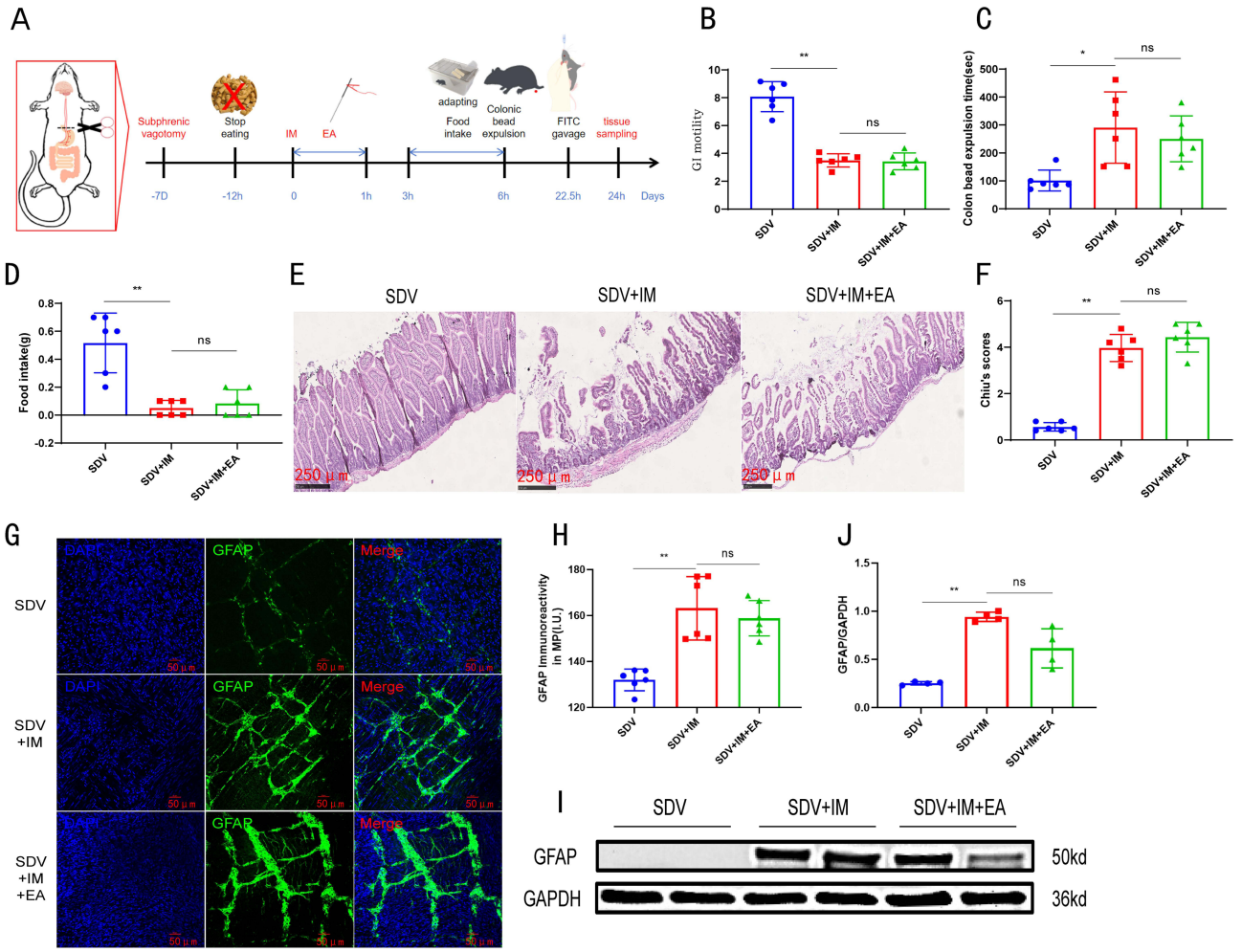

Figure 6 Loss of EA protection after SDV. (**A**) Schematic representation of the experimental protocol. (**B**) The mean GI transit at the 24th hour after surgery (n=6). (**C**) Colonic bead expulsion time at the 6th hour after surgery (n=6). (**D**) Food intake from the 3rd hour to the 6th hour after surgery (n=6). (**E** and **F**) HE staining and Chiu's scores of small intestine (n=6). (**G** and **H**) Changes of GFAP expression in intestinal myenteric plexus by IF staining (n=6). (**I** and **J**) Changes of GFAP expression in the small intestine by WB (n=4). One-way ANOVA test with post-hoc test * $P < 0.05$, ** $P < 0.01$, $P > 0.05$.
Abbreviation: ns, not significant.

sympathetic hyperexcitability, alleviating the suppression of GI function caused by postoperative stress response,[44] thereby reducing visceral vasoconstriction and optimizing GI mucosal perfusion and nutrient absorption efficiency.[45] Notably, EA ST 36 may also regulate appetite-related nuclei[46,47] such as the hypothalamic arcuate nucleus and nucleus accumbens, upregulating the expression of orexigenic factors like neuropeptide Y[48] while suppressing anorectic mediators such as leptin,[42] thus enhancing feeding behavior. Additionally, the improvement in gut microbiota diversity may synergistically promote nutrient digestion and absorption.[49] Although mechanistic evidence in mice requires further validation, clinical studies demonstrating EA's alleviation of postoperative abdominal distension and nausea support indirectly its potential effect.[50,51]

The vagus nerve, as a crucial bridge between central nervous system and GI tract, plays a critical role in EA ST 36-mediated recovery of GI function.[52] Experimental data show that EA ST 36 significantly increases the amount of c-Fos protein in NTS and elevates plasma ACh levels, reversing the suppression of vagal activity induced by IM. Study has confirmed that optogenetic activation PROKR2^{Cre}-marked sensory neurons at ST 36 enhances the expression of Fos^{+}ChAT^{+} neurons in the dorsal motor nucleus of the vagus, driving the neuro-adrenal axis and reducing TNF and IL-6 by up to 50%.[26] Quantitative analysis of HRV parameters (RR interval, HF, LF/HF ratio) further confirms that EA ST 36 restores vagal tone and ameliorates IM-induced autonomic imbalance. Using models of systemic vagal blockade (LCV) and GI-specific vagal denervation (SDV), it was found that vagotomy abolished the therapeutic effects of EA on

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GI propulsion and EGCs reactivity. Collectively, EA ST 36 promotes postoperative GI motility recovery by activating the vagal pathway and modulating autonomic balance, with its mechanism attributed to the systemic regulation of the neuro-immune-endocrine network.

The abnormal activation of reactive EGCs is closely associated with postoperative GI dysfunction.[9] Studies indicate that surgical incision alone triggers EGCs activation, and subsequent surgical interventions further exacerbate their transition to a reactive EGC state.[53] Our data corroborates this phenomenon, as IM-induced POI significantly elevated GFAP fluorescence intensity and expression, while EA ST 36 effectively inhibited this pathological process. Notably, the mAChR agonist carbachol reduced GFAP expression and attenuated EGCs activity, improving GI hypomotility. This finding aligns with recent studies demonstrating that EA ST 36 inhibits EGCs activity and S100β release through a noradrenergic pathway, alleviating visceral hypersensitivity in ulcerative colitis models.[54] Although other studies have reported bidirectional regulation of EGCs by EA, such as activating EGCs via α7 nicotinic ACh receptors to improve intestinal barrier function in hemorrhagic shock[28] or promoting EGCs autophagy through the PI3K/AKT/mTOR pathway,[55] our experiments indicate that EA ST 36 exerts therapeutic effects in the POI model by suppressing EGCs reactivity. These differences may be related to the functional plasticity of EGCs under different pathological conditions, as well as variations in EA stimulation parameters and acupoint selection.

Muscarinic receptor-mediated cholinergic signaling is a core mechanism regulating EGCs function. This study revealed that: EA ST 36 upregulated m3AChRs expression in EGCs of POI mice, accompanied by the recovery of GI motility; The m3AChR antagonist J104129 blocked the protective effects of EA; The m3AChR agonist carbachol mimicked the effects of EA by inhibiting EGCs reactivity. IF co-localization demonstrated that m3AChRs co-localizes with EGCs markers GFAP and exhibited synchronous changes, suggesting that EA directly modulated EGCs function through the vagus nerve-ACh-m3AChR axis. This result aligns with the findings of Delvalle et al, showing that m3AChRs are expressed on the surface of EGCs, and their activation enhances intestinal motility by increasing intracellular Ca^{2+} activity.[17] In vitro colon experiments further support this mechanism: selective activation of m3AChRs on EGCs increases the amplitude and frequency of colonic migrating motor complexes, promoting colonic motility.[56]

IF co-localization results show that m3AChRs are expressed on the surface of EGCs and exhibit co-localization signals with EGCs markers GFAP. Although m3AChRs fluorescence is also distributed around enteric neurons, this finding suggests that the regulatory effects of EA ST 36 on EGCs are at least partially mediated through m3AChRs on their surface. Further experiments reveal that changes in m3AChRs occur synchronously with the EGC marker GFAP, indicating a functional association between the two. Therefore, based on these, we propose that vagal efferent signals may mediate the interaction between neurons and EGCs through paracrine pathways, coordinating EGCs reactivity to regulate GI motility and inflammation. This bidirectional communication involves multiple signaling molecules[57] (eg, purines, γ-aminobutyric acid, ACh) and is achieved through the synaptic/non-synaptic structures of EGCs, which are highly sensitive to neuronal activity and can bidirectionally regulate GI motility, secretion, and barrier function.[58] For example, Boesmans et al[18] found that optogenetic activation of enteric neurons induces synchronized Ca^{2+} transients in adjacent EGCs, while P_2 receptor antagonists (suramin, and pyridoxal phosphate-6-axophenyl-2′-4′-disulfonic acid) significantly reduce EGC responses, indicating that purinergic signaling mediates neuron-EGC communication. Additionally, EGCs may be involved in the formation of complex axons in enteric neurons and regulate axon density, which may be mediated by the P_2Y_1R- and GDNF-dependent pathways,[59] since neutralization of GDNF and blockade of P_2Y_1R abolishes this regulatory effect. This conjecture of ours is consistent with the description of Gonzales et al.[57] Notably, the expression of muscarinic signaling pathways in enteric neurons and EGCs synergistically regulates GI motility, while ACh can also inhibit inflammatory responses through α7 nicotinic ACh receptors,[32] highlighting the pleiotropic role of cholinergic signaling in GI homeostasis.

While our study provides mechanistic insights, several limitations merit consideration. First, we did not systematically investigate ST 36's interaction with vagal afferent pathways, which reflects our focused exploration of efferent vagal signaling. Second, the current design cannot conclusively delineate whether EA-mediated EGCs modulation occurs via enteric neurons or direct m3AChR activation on EGCs, though our data substantiate m3AChRs' partial contribution. Crucially, downstream EGCs signaling cascades was not further investigated, and this needs to be refined by further experiments. For instance, utilizing cell-type-specific transgenic models to generate conditional m3AChR knockouts in neuronal and/or EGC populations, combined

with advanced functional imaging techniques (eg, calcium imaging) to simultaneously monitor bidirectional neural-EGC signaling dynamics.

Our findings demonstrate that m3AChR activation mediates ACh-dependent suppression of EGCs reactivity, which restores vagally regulated GI tract motility. Experimental data revealed that EA attenuates IM-induced EGCs activation via m3AChR signaling, an effect abolished by the selective m3AChR antagonist J104129 and vagotomy. Mechanistically, m3AChRs expression in myenteric plexus ganglia critically mediates EA's therapeutic efficacy against POI.

Conclusion

As far as we know, this is the first study in POI to show that EA ST 36 regulates reactive EGCs via m3AChRs to ameliorate GI motility dysfunction. In this study, we found that EA ST 36 improves postoperative GI motility dysfunction in POI mice by activating the vagus nerve to release ACh and regulating reactive EGCs by m3AChRs (Figure 7). In summary, our study provides strong evidence to support the efficacy of EA treatment in alleviating GI motility dysfunction in POI mice. It is suggested that EA ST 36 is a promising treatment for POI as a non-invasive and effective treatment.

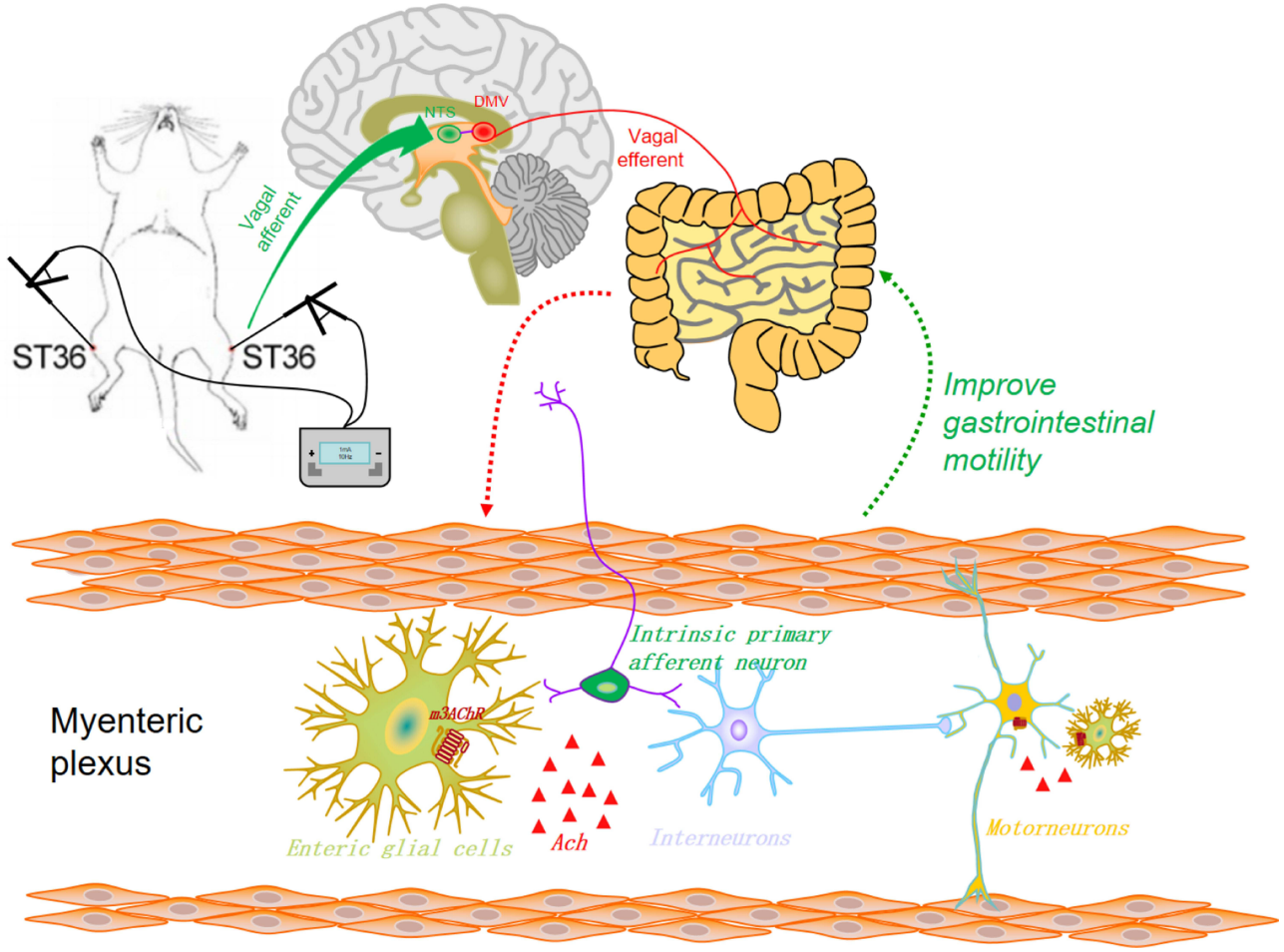

Figure 7 A schematic representation of the potential mechanisms by which EA ST 36 improves postoperative GI dysfunction. Specifically, EA ST 36 activates the vagus nerve, and vagus nerve efferents regulate EGCs reactivity through m3AChRs to improve postoperative GI motility dysfunction in POI mice. Solid green arrows: stimulation at ST 36 is introduced to NTS through vagal afferent; Red dotted arrows: vagal efferent signals to intestinal myenteric plexus; Green dotted arrows: intestinal myenteric plexus react to improve GI motility.

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Abbreviations

ACh, acetylcholine; CNS, central nervous system; DMV, dorsal motor nucleus of the vagus; EA, electroacupuncture; EGCs, enteric glial cells; ENS, enteric nervous system; FC, fluorocitrate; GI, gastrointestinal; GFAP, glial fibrillary acidic protein; HRV, heart rate variability; IF, immunofluorescence; IM, intestinal manipulation; LCV, left cervical vagectomy; LMMPs, longitudinal muscle and myenteric plexuses; m3AChRs, type 3 muscarinic acetylcholine receptors; NTS, nucleus tractus solitarius; POI, postoperative ileus; SDV, subphrenic vagectomy; ST 36, Zusanli acupoint; VNS, vagus nerve stimulation; WB, Western blot.

Data Sharing Statement

All data analyzed in this study are available from the corresponding author at 304637372@qq.com.

Ethics Statement

All experimental procedures and animal surgeries were conducted in accordance with the protocol approved by the Experimental Animal Ethics Committee of Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangdong Academy of Chinese Medicine (Approval No. 2024005). Animal welfare was in accordance with Laboratory animal-Guideline for ethical review of animal welfare (GB/T 35892–2018). All experimental mice were purchased from Rise Mice Biotechnology Co., Ltd. [license number: SCXK (yue) 2020-0053].

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Disclosure

The authors report no conflicts of interest in this work.

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