Reactive Enteric Glial Cells Participate in Paralytic Ileus by Damaging Nitrergic Neurons During Endotoxemia

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Research Article

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Abstract

Background

Paralytic ileus is common in patients with septic shock, which may cause high morbidity and mortality. Enteric neurons and enteric glial cells (EGCs) participate in the regulation of intestinal motility, but little is known about their role. We aimed to prove whether reactive EGCs have harmful effects on enteric neurons during endotoxemia and lead to intestinal motility disorder in mice.

Methods

In this study, lipopolysaccharide (LPS) was used to induce endotoxemia in mice, and intraperitoneal injections of fluorocitrate (FC) twice per day (9 AM and 6 PM) for 7 days before LPS injections to prevent the activation of EGCs. The effects of reactive EGCs on intestinal motility were analyzed by motility assays *in vivo* and colonic migrating motor complexes (CMMCs) *in vitro*. The changes of enteric neurons were evaluated by immunofluorescent staining HuCD, nNOS, CHAT, and TUNEL.

Results

The expression of glial fibrillary acidic protein (GFAP) was significantly upregulated in LPS-injected animals, indicating EGCs were transformed into a reactive state. The administration of FC could significantly prevent it. Meanwhile, inhibition of reactive EGCs can improve intestinal motility and peristaltic reflex. The density of the general neuronal population (HuC/D-immunoreactive) in the colonic myenteric plexus was significantly increased after suppressing reactive EGCs. The population of nNOS neurons was increased significantly, but there was no significant difference in the number of ChAT neurons. Furthermore, the apoptotic rate of enteric neurons significantly increased, when incubated with the conditional medium of reactive EGCs *in vitro*. At the same time, the dendritic complexity and the number of primary neuritis neurons were significantly reduced.

Conclusion

Reactive enteric glial cells participated in paralytic ileus by damaging nitrergic neurons during endotoxemia. It may provide a novel therapeutic strategy for intestinal motility disorders during endotoxemia or sepsis.

Introduction

Sepsis is a life-threatening organ dysfunction caused by the host's dysregulated response to infection with roup to 26% mortality in the past 10 years[1]. Intestinal motility disorder often manifests as paralytic ileus, which is a common complication following sepsis and septic shock. It can cause bacteria accumulation in the intestine, and promote bacterial translocation. In turn, it can induce multiple organ dysfunction syndromes (MODS) and lead to high mortality[2]. Thus improving intestinal motility is critical during sepsis treatment[3].

Intestinal motility is regulated by the enteric nervous system (ENS), a complex network of neurons and glial cells, mainly resides in the submucosal and myenteric plexus[4]. There is evidence that dysfunction, degeneration, or loss of myenteric neurons can cause several intestinal dysmotility disorders [5, 6]. On the other hand, enteric glial cells (EGCs) are abundant cells in the ENS, which can provide nutritional support for enteric neurons, promote the formation and function of synapses, and maintain neuronal survival[7].

EGCs may undergo a dramatic transformation called the “reactive glial phenotype” in response to intestinal infection with bacteria[8], viruses and inflammatory mediators[9, 10]. However, there is controversy regarding the function of reactive EGCs. Previous studies have shown EGCs may have dual protective and harmful effects. For instance, the intestinal ischemia-reperfusion injury (IRI) leads to the significant upregulation of glial fibrillary acidic protein (GFAP), the EGC activation marker. That presents a beneficial effect on neurons through the increase of glial cell line-derived neurotrophic factor (GDNF) under this condition[11, 12]. On the contrary, a study from Gulbransen's group provided evidence that glial activation as a driver of enteric neurodegeneration to induce the death of enteric neurons through purinergic pathways in colitis induced by Dinitrobenzene sulfonic acid (DNBS) [13]. As for endotoxemia, it has been confirmed that lipopolysaccharide (LPS) increase induces proinflammatory cytokines release and myenteric neurons apoptosis in vitro[14]. In addition, Systemic LPS administration can also induce an increase in intestinal GFAP and S100b expression[15]. While the association between reactive EGCs and the loss of enteric neurons is still unknown. That is to say, it is unclear whether reactive EGCs are beneficial or harmful to influence the enteric neurons in endotoxemia mice.

In this study, we investigated the effect of reactive EGCs on intestinal motility and enteric neuron function in an endotoxemia model *in vivo*, and observed the effect of reactive EGCs on the growth and survival rate of enteric neurons under an inflammatory condition *in vitro*. Here we observed that LPS-induced reactive EGCs are harmful to the enteric neurons during endotoxemia and are associated with colonic transit delay, which may be used to prevent and treat LPS-induced intestinal paralysis and other relevant intestinal motility disorders.

Materials And Methods

1. Animals

C57BL/6 mice (male, 6~8 weeks old, weight 20~23g) were obtained from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China) and housed in a temperature-controlled and humidity-controlled room with a 12:12 hour light: dark cycle, and provided with food and water. All procedures were approved by the Institutional Animal Care and Use Committees at Xi'an Jiaotong University (Xi'an, China). Animals were randomly divided into 3 groups: Con: vehicle control, normal saline (0.02ml/g, IP), LPS: lipopolysaccharide (E. coli O55:B55, Sigma-Aldrich, USA, 20mg/kg, IP), LPS+fluorocitrate (FC): LPS injection after intraperitoneal injections of FC (Sigma-Aldrich, USA, 20μmol/kg) twice per day (9 AM and 6 PM) for 7 days according to the previous research[16] (Supplementary Figure S1). Animals were observed

and evaluated at least twice a day for post-treatment care until sacrifice, including breath, body temperature and activity.

2. Assessment of gastrointestinal motility

2.1 Stool frequency and fluid content

According to the previous method[17], each mouse was placed in a clear cage for 1hr. Fecal pellets were collected, counted, and weighed (wet weight) during this time. These were dried overnight at 60℃ and weighed again (dry weight). Fluid content (%) =100% × (wet weight-dry weight)/wet weight.

2.2 Colon Bead Assay

Distal colon transit was assessed using glass beads (2mm in diameter) as previously described[17], and executed 48hrs after the LPS injection. After being overnight fasted, mice were anesthetized with inhalation of isoflurane and a single bead was inserted through the anus and pushed 2cm aborally by a customized needle with a silicon cannula (1.9mm in diameter) (Cat. No. 9921, Cadence Inc., Staunton, VA). The needle was then withdrawn and the bead expulsion latency was obtained. The time required to eject the bead was measured as an estimate of colonic motility.

2.3 Whole-Gut Transit

Whole intestinal transit time was measured following the previous report with a little modification[18] and executed 48hrs after the LPS injection. Male mice (8 weeks) have fasted only with access to water for 18 hrs before the experiment. 0.2mL of a solution containing 6% (w/v) Carmine (Cat. No. C1022, Sigma-Aldrich Co., St Louis, MO) and 0.5% (w/v) methylcellulose (Cat. No. M0262, Sigma-Aldrich Co.) dissolved in ultrapure water was orally administered to each mouse and were left undisturbed in individual cages with food and water ad libitum. 2 hrs after gavage, pellets coloration was checked regularly every 20 mins. Time elapsed from gavage until the appearance of the first red pellet was obtained.

2.4 Colonic Migrating Motor Complexes Measured *in vitro*

According to the previous method[19], each colon was dissected from the mouse and flushed to remove fecal content with Krebs solution. Then the empty colon was mounted in a horizontal organ bath with oxygenated Krebs solution at 35°C and maintained intraluminal pressure at 2cmH_2O. Preparations were equilibrated for 30 mins and four 15-minute videos of contractile activity were captured via a video camera (Logitech, Newark, CA) positioned 7~8cm above the gut. Finally, these videos were converted to spatiotemporal maps via MATLAB (MathWorks, USA). The frequency of colonic migrating motor complexes (CMMCs) as well as the velocity and length of their propagation were analyzed by a researcher blinded to the test groups.

3. Immunofluorescent staining

3.1 Evaluation of HuC/D-, ChAT- and nNOS-immunoreactive myenteric neurons

Whole-mount longitudinal muscle/myenteric plexus (LMMP) preparations of colonic myenteric plexus were prepared according to a published method in Tricia H. Smith et al[20]. After washing these in phosphate-buffered saline, colon LMMP preparations were fixed in 4% formaldehyde 1hr and then permeabilized with 0.3% Triton-X for 40mins before immunostaining. Preparations were blocked with 10% goat serum for 1hr and exposed to primary antibodies used to detect the general population of myenteric neurons (mouse anti-HuC/D, 1:100; Invitrogen), subpopulation of cholinergic neurons (goat anti-choline acetyltransferase, ChAT, 1:200; Millipore) and nitrergic neurons (rabbit anti-neuronal nitric oxide synthase, nNOS, 1:200; GeneTex). Preparations were incubated with primary antibodies for 16-24hrs, visualized with species-specific secondary antibodies (Alexa Fluor 350, 488, or 594; 1:500) and mounted. Images of the myenteric ganglia were taken under the confocal microscope. ImageJ 1.52 was used to analyze neuronal density in the myenteric ganglia. In each field, the number of HuC/D-immunoreactive neuronal bodies and nNOS neurons per area (cells/mm^2 ganglionic area)was manually counted in a blind fashion. A computer-controlled motorized stage was used to scan images (20× objective) covering a $10mm^2$ area. At least, 5 images that were captured with a 20× objective were counted from each animal (n = 6, each group). All the studies were performed in a double-blind fashion. Moreover, the intensity fluorescence of ChAT was detected by ImageJ, along with several adjacent background readings. The CTCF (corrected total cellular fluorescence) [=integrated density − (area of selected cell × mean fluorescence of background readings)] was calculated[21].

3.2 Fluorescence analysis of glial fibrillary acidic protein

GFAP (1:100, # GTX85454; GeneTex, USA) immunolabeling was performed using colon LMMP preparations to assess the fluorescence intensity. The images were captured using the same high-resolution camera and fluorescence microscope. The exposure brightness, contrast, and time were maintained for each photomicrograph. ImageJ 1.52 was used to measure their intensity.

4. Western blotting

Protein samples were extracted from colonic tissue in RIPA buffer containing complete protease and phosphatase inhibitor cocktail (Roche). After assessing the protein content of each sample by BCA Protein Assay kit (#23227, Pierce), they were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. After blocking, the blots were incubated with an anti-GFAP (1:10000, # GTX100850; GeneTex, USA) primary antibody overnight at 4°C. Subsequently, the membrane was incubated with a secondary antibody (anti-rabbit IgG-horseradish peroxidase, 1:2000, #GTX213110-01; GeneTex, USA) followed by visualized with enhanced chemiluminescence (Merck Millipore, USA). β-actin was used as an internal standard. Immunoblots were quantified by NIH Image J software.

5. Cell cultures

5.1 Primary enteric glial cells

The isolation, identification, and culture of primary EGCs were performed as previously described[22]. Newborn rats (Sprague Dawley strain) were deeply anesthetized by isoflurane and decapitated. In brief, colonic tissues were rinsed in Hank's balanced salt solution (Hank's balanced salt solution, HBSS, Gibco Life Technologies), and then separated the LMMP from the underlying circular muscle. Use scissors to snip LMMP into tiny pieces and place them into digestion solution, trypsin (0.1mg/ml; Gibco Life Technologies), incubated for 15mins at 37˚C, and then centrifuged at 900 rpm. The trypsin reaction was stopped by adding fetal calf serum (Gibco Life Technologies). Cells suspended into DMEM-F-12 (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1mM glutamine and 100IU/ml penicillin/streptomycin, 20μg/ml Gentamicin and 2mM L-Glutamine were plated on Poly-D-Lysine-coated (0.01%; Sigma Aldrich, USA) 6 wells. The cultures were kept in a humidified atmosphere of 95% air/5%CO2 at 37˚C, changing half of the cell media every 2days. Glial cells were passaged to coverslips until the cell attachment rate was up to 90%. Culture consisted of approximately 90% of enteric glia, as judged by immunolabelling with a chicken antibody specific for GFAP and rabbit antibody for S100b protein.

5.2 Primary enteric neurons

The isolation and culture of enteric neurons are similar to that of primary EGCs. The difference is that the colonic tissues were collected from embryonic day 15 (E15) rats, and cells were incubated in a neurobasal medium containing 2 mM glutamine, 1 mM (100 IU/ml) penicillin/streptomycin, and 1 mM B-27 supplement. The purity of neurons is also more than 90% through immunofluorescence staining.

5.3 Enteric glial cell line

The rat enteric glial cell line CRL2690 (American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1mM glutamine, and penicillin/streptomycin.

6. Conditioned medium experiments

Because LPS triggers immune responses in endotoxemia, leading to a massive release of cytokines such as IL-1β and TNF-α[3, 23], therefore, we induced the reactive EGCs by IL-1β and TNF-α *in vitro* to mimic the inflammatory response induced by LPS *in vivo*. IL-1β (80ng/ml, Peprotech, USA) and TNFα (60pg/ml, Peprotech, USA) were used to induce EGCs into reactive for 24hrs according to the previous study[22], then the medium was removed and replaced with a fresh neurobasal medium for another 24hrs to generate EGCs conditioned medium (ECM). This was added to primary enteric neurons in 24 wells for 24hrs. Finally, neurons were fixed and prepared for the next experiments as described below.

6.1 Neurite formation assay

Primary enteric neurons were plated at 1,000 cells/well and grown for 12 days in the neurobasal medium. They were treated with the conditioned medium of control and reactive EGCs for 4 days. For analysis of neurite formation, enteric neurons were fixed and stained with antibodies against the neuron-specific

class III β-tubulin (Tuj1). Sholl analysis was used to assess the complexity of neurites. Pictures of Tuj1-labeled cells were acquired with 20× objective and a template of concentric circles distant from 10 to 500 μm (10 μm interval) from the ganglion center was overlaid on the ganglion using Image J software. The number of primary neurites and branching points was also counted. For each group (n = 6), eight ganglia were analyzed.

6.2 Detection of apoptosis

To detect apoptosis, TUNEL staining was carried out using the fluorescein tagged In Situ Cell Death kit (Promega, USA) according to the procedures of specification. Primary enteric neurons were plated at 4×10^4 cells/well in 24-well plates and grown for 7days after maturing in the neurobasal medium. They were treated with the conditioned medium of control and reactive EGCs. 48hrs after the treatment, neurons were fixed in 4% paraformaldehyde (PFA) for 20mins at 25^{o}C and then were cleared of fixative with 3×5mins washes in PBS. The next steps were according to the procedures of the specification.

7. Statistical Analysis

Results are presented as mean ± Standard Error of Mean (SEM). All statistical analysis was conducted using Prism 7.0 (GraphPad, San Diego, CA, USA). The Student's t-test was used to determine statistical differences between each experimental group and the control group data. One-way ANOVA with Dunnett's multiple comparisons was used for the data with group numbers over two. $P < 0.05$ was considered significant and denoted by *.

Results

1. Reactive EGCs were induced whereas were reversed by FC in LPS-injected animals

GFAP is a typical identification marker of enteric glia and astrocytes to indicate their activation and proliferation, whose expression was significantly upregulated after the LPS injection compared with the control group (Fig. 1a and b, $P < 0.01$). FC is a metabolic compound that makes EGCs metabolism static [16]. To testify whether EGCs were no longer reactive in the mice treated with 20μmol/kg FC twice daily for 7days, we injected LPS and assessed the protein expression of GFAP 48hrs later. Western blot analysis showed that GFAP expression levels in colon tissues were decreased significantly compared to the LPS group ($P < 0.001$), similar to the control one ($P > 0.05$). To eliminate the effect of mucosal EGCs, we isolated the LMMPs from the underlying circular muscle and detected the expression intensity of GFAP in the myenteric nerve plexus by immunostaining. It had the same trend as the result of the western blot (Fig. 1c-f). It indicated that EGCs converted into a reactive state, while, FC impeded this produce.

2. Inhibition of reactive EGCs improved intestinal motility and peristaltic reflex in LPS-injected animals

To determine whether the reactive EGCs are involved in the regulation of GI motility, FC was employed to make EGCs metabolism static. Then, pellet output, propulsive colorectal motility, total gastrointestinal

transit time (TGIT), fluid content *in vivo was* measured. LPS significantly reduced gastrointestinal motility, while FC plus LPS tended to reverse it back obviously(Stool frequency, mean control 8.833 ± 0.8333, vs. LPS 2.5 ± 0.4282, Fig. 2a, $P < 0.01$, colon transit time, mean control 49.83 ± 9.631s, vs. LPS 1044 ± 235.1s, Fig. 2b, $P < 0.001$, TGIT, mean control 72.5 ± 4.61min, vs. LPS 541.2 ± 63min, Fig. 2c, $P < 0.0001$, n=6). Stool frequency(mean FC+LPS 5.333 ± 0.5578 vs. LPS group, Fig. 3a, $P < 0.05$, n=6), colon transit time(mean FC+LPS 188.8 ± 60.35, vs. LPS group, Fig. 3b, $P < 0.01$, n=6) and TGIT(mean FC+LPS 201.3 ± 44.18min vs. LPS group, Fig. 3c, $P < 0.01$, n=6) were significantly alleviated in the FC treatment group, comparing to the group only with LPS injection group. There was no statistically significant difference in stool water content among the three groups (mean control 0.6313 ± 0.03698, vs. LPS 0.6125 ± 0.03581, vs. FC+LPS 0.4799 ± 0.06607, Fig. 2d, $P > 0.05$, n=6).

CMMCs, which are dependent on ENS [24], were investigated in the isolated preparations of colons to determine whether the observed changes in motility are due to an intrinsic ENS defect. Spatiotemporal maps of CMMCs were constructed and analyzed (Fig. 2e-g), showing that frequency (mean control 1.15 ± 0.1912CMMCs/min, vs. LPS 0.361 ± 0.06351CMMCs/min, Fig. 2h, $P < 0.001$, n=6) and velocity (mean control 22.95 ± 3.902mm/min, vs. LPS 5.297 ± 0.3616mm/min, Fig. 2i, $P < 0.001$, n=6) were significantly decreased in the LPS group compared with the control group. However, both of them back to normal when treated with FC(frequency, mean FC+LPS 0.7794 ± 0.03554 CMMCs/min vs. LPS, $P < 0.05$, velocity, mean FC+LPS 17.27 ± 1.146 mm/min, vs. LPS, $P < 0.05$, n=6). To sum up, the reactive EGCs slow intestinal transit and disrupt the peristaltic reflex, while FC can mitigate this deficiency.

3. Inhibiting reactive EGCs reduced the loss of nitrergic neurons in the myenteric plexus of LPS-injected animals

To study how reactive EGCs affect enteric neurons in LPS-injected animals, the density of general neuronal population (HuC/D-immunoreactive) in the colonic myenteric plexus was accessed among the control, LPS, and LPS plus FC group. In line with the literature[25], LPS reduced the number of general neurons by 28% compared with the control mice (mean control 662.85 ± 55.71/mm^2, vs. LPS 476.19 ± 62.22/mm^2, Fig. 3a and b). Obviously, the neuronal population did not decrease in the FC plus LPS group, but increased significantly compared with the LPS group (mean FC+LPS, 719.05 ± 50.16/mm^2, vs. LPS group, $P < 0.05$). Furthermore, to define whether the reactive EGCs also affected the neurochemical coding profile of the myenteric neurons, ChAT and nNOS expression in the colonic myenteric plexus was measured using immunofluorescence staining. LPS reduced the population of nNOS neurons per square millimeter of colonic LMMPs (mean control 292.4 ± 27.82/mm^2, vs. LPS 156.4 ± 24.62/mm^2, $P < 0.05$), which reversed to baseline under the condition of EGC ablation (mean FC+LPS 251.4 ± 23.39/mm^2, vs. LPS group, Fig. 3c, $P < 0.05$). Although there was no significant difference in the ChAT fluorescence intensity among these groups (Fig. 3d, $P > 0.05$). These observations demonstrated that the reactive EGCs cause enteric neuron loss in LPS-injected animals.

4. Reactive EGCs increased neuronal apoptosis *in vitro* in primary enteric neurons

It has been testified that reactive EGCs lead to a decrease of enteric neurons in the myenteric plexus. Furthermore, we studied how reactive EGCs influence the state of enteric neurons *in vitro*. First, we used IL-1β and TNFα to build a serum-free culture model for the reactive EGCs *in vitro,* mimicking the pathological process in LPS-injected animals (Fig. 4a-e). And then, purified enteric neurons were incubated with the conditional medium of resting or reactive EGCs. Then the cell apoptosis was assessed using TUNEL tests and cleaved Caspase-3 protein staining. The TUNEL assay showed that primary enteric neurons revealed 27.54% ± 5.28 positive apoptotic neurons at 48hrs after cultured with the conditional medium of reactive EGCs compared to 3.06% ± 0.34 for control cultures (Fig. 5a and b, $P < 0.01$). Immunofluorescence of cleaved Caspase-3 protein displayed that 6.84% ± 0.35 positive apoptotic neurons cultured with the conditional medium of reactive EGCs compared to 3.46% ± 1.16 for control neurons (Fig. 5c and d, $P < 0.01$).

5. Reactive EGCs disrupted neurites formation of primary enteric neurons *in vitro*

To test if reactive EGCs affect neurites formation of enteric neurons *in vitro*, primary enteric neurons were incubated with the medium of resting or reactive EGCs and observed through TuJ1 immunostaining (Fig. 6a). The dendritic complexity of neurons cultured in reactive EGCs conditional medium significantly declined compared to those grown in the resting EGCs conditional medium (Fig. 6b, $P < 0.05$). In addition, the primary neuritis number of neurons cultured in reactive EGCs conditional medium decreased by 54.17% (+resting EGCs 6 ± 0.5774, vs. +reactive EGCs 2.75 ± 0.8539, Fig. 6c, $P < 0.05$) and the number of branches also reduced by 77.14% (+resting EGCs 19.25 ± 1.031, vs. +reactive EGCs 4.4 ± 0.8718, Fig. 6d, $P < 0.0001$). Neurons cultured in reactive EGCs conditional medium tend to show shorter neurites compared to those grown in the resting EGCs conditional medium (+resting EGCs 141.8 ± 30.22, vs. +reactive EGCs 72.03 ± 20.21, Fig. 6e, $P > 0.05$), However, there was no significant difference between them (Supplementary Figure S2).

Discussion

In this study, we demonstrated that reactive EGCs caused a significant decrease in intestinal motility via increasing the apoptosis of nitrergic enteric neurons in LPS-injected animals. However, this tendency can be reversed, once the function of reactive EGCs was inhibited by FC. In addition, reactive EGCs also disrupted the neurites formation of primary enteric neurons *in vitro*. Our results indicate that the reactive EGCs may cause damage to the enteric neuron during endotoxemia, which deteriorates the intestinal motility disorder, indicating that inhibition of the reactive EGCs may be a therapeutic strategy for the treatment of LPS-induced intestinal paralysis and other intestinal motility disorders.

Early research showed that EGCs play an important role in regulating and protecting enteric neurons, and dysfunction of EGCs will break the intestinal homeostasis, leading to a series of gastrointestinal disorders[26]. Furthermore, previous studies demonstrate that enteric glia could be activated by bacteria, which will respond to an immune reaction in the gastrointestinal tract and release ATP[13], inflammatory cytokines[27], and prostaglandin, etc[28]. However, whether the reactive EGCs participate in

neurodegenerative processes and whether the interaction between EGCs and neurons influences intestinal motility is not clarified yet. In order to elucidate this question, we tested the intestinal motility of mice injected LPS and FC plus LPS, as well as the number of colonic LMMP neurons. Compared to the control, there was a significant delay of intestinal transition accompanied by a significant loss of neurons after the LPS injection. In contrast, the result showed that inhibition of EGC activation could improve intestinal motility and prevent neurons from losing. Thus, we inferred that the reactive EGCs were associated with the decrease of enteric neurons. Meanwhile, it has been proved that ENS plays a significant role during this process[29] and the loss of enteric neurons causes intestinal dysmotility[6]. Therefore, if we could impede the reactive EGCs, the loss of neurons may be alleviated as well as the intestinal dysmotility.

EGCs were primarily considered as a component to provide structural support for the enteric neural net. Currently, it has been confirmed that EGCs could release nerve growth factor (NGF), neurotrophin 3 (NT-3), and GDNF to maintenance and contribute to the survival of enteric neurons[30, 31]. Interestingly, glia transforms into a reactive phenotype in response to intestinal infection with bacteria or viruses, inflammatory mediators[32]. The function of reactive EGCs, though unlike the central nervous system (CNS), is still poorly understood. As shown for the CNS, 2 different types of reactive astrocytes termed “A1” and “A2” were induced by neuroinflammation and ischemia respectively[33]. Furthermore, A1 reactive astrocytes become neurotoxic, leading to the neuron’s death[34]. As for ENS, the intestinal ischemia-reperfusion injury (IRI) leads to the significant upregulation of GFAP, which may present a beneficial effect on neurons, because GDNF was increased under this condition [11, 12]. On the contrary, a study from Gulbransen’s group provided evidence that glial activation is a driver of enteric neurodegeneration in colitis[13]. Besides, Andromeda’s group used human EGCs from GI-surgical specimens and treated them with LPS to study the gene expression and relevant functions. Their results showed that the “reactive human EGC phenotype” leads to alterations of important molecular and functional signaling pathways that could disrupt GI motility[35]. After all, there is still a lack of study about the influence of reactive EGCs on enteric neurons. In our study, we used a conditional medium of reactive EGCs to interfere with primary enteric neurons, the results showed that the reactive EGCs damaged enteric neurons through increasing neurons’ apoptosis and disrupting neurites’ formation.

Because we used a conditional medium of reactive EGCs, so it suggested that EGCs might release some specific substances to be harmful to neurons. Thus, in the following study, we can try to find such potential substances or targets that reactive EGCs damage enteric neurons and block them, which may be a potential new therapeutic target to modulate the intestinal dysmotility of patients suffering from sepsis or endotoxemia, or other motility disorders. We also cannot entirely exclude the possibility that the reactive EGCs communicate with neurons via other approaches, such as Calcium waves. It still needs to be measured in the future.

As a first attempt to characterize the reactive glial phenotype, GFAP was used to reveal it. GFAP, a glial fibrillary acidic protein, is the typical identification marker of enteric glia and astrocytes, whose expression

in mature EGCs is modulated by cell differentiation, inflammation, and injury[36]. Increased GFAP has been observed in ulcerative colitis, Crohn's disease[26], and Parkinson's disease[37]. Astrogliosis and EGCs can be induced by cytokines and LPS[38, 39]. To study some functions of EGCs in the endotoxemia model, we choose LPS *in vivo*. There was an upregulation of the GFAP expression in the LMMP of colon tissues in line with another research[15].

FC was chosen to block the effect of LPS on the enteric glia. It can be selectively taken up by enteric glia in the intestine [40], which causes a reversible and selective disruption of glial function owing to a fall in glia ATP levels through inhibiting the tricarboxylic acid TCA cycle, but does not influence the morphology of glia or neuron, being consistent with an immunological method of glial ablation [16]. In our research, when FC disrupted the function of EGCs, LPS did not inhibit gastrointestinal motility and the number of enteric neurons was not decreased anymore. Thus, the reactive EGCs appear to have a harmful effect on intestinal motility in LPS-injected animals.

There is still a phenomenon that cannot be elucidated—we found that the amount of total and nitrergic neurons in the myenteric nerve clusters was decreased, except for cholinergic neurons, which is similar to a previous study[5]. In view of other research, there are several types of enteric neurons in ENS, in which cholinergic neurons and nitrergic neurons are mainly involved in intestinal motility regulation[41]. Cholinergic neurons, in theory, mediate extrinsic nerve input, however, nitrergic neurons are inhibitory motor ones[42]. It may be relevant to the interaction and coordination between different kinds of enteric neurons, and the broken balance of intestinal neural networks might cause intestinal disorders. All these need further research.

Conclusions

In conclusion, the present data indicated that reactive enteric glial cells were involved in paralytic ileus through promoting nitrergic enteric neuronal apoptosis and impeding the formation of neurites enteric neurons during endotoxemia. These findings may provide a novel therapeutic strategy for intestinal motility disorders, including LPS-induced intestinal paralysis. In addition, the specific mechanism about how the reactive EGCs damage enteric neurons needs further study.

Abbreviations

EGC	Enteric glial cell
LPS	Lipopolysaccharide
FC	Fluorocitrate
CMMC	Colonic migrating motor complexe
GFAP	Glial fibrillary acidic protein
MODS	Multiple organ dysfunction syndromes
ENS	Enteric nervous system
IRI	Ischemia-reperfusion injury
GDNF	Glial-derived neurotrophic factor
DNBS	Dinitrobenzene sulfonic acid
LMMP	Longitudinal muscle/myenteric plexus
CTCF	Corrected total cellular fluorescence
FBS	Fetal bovine serum
TuJ1	Anti-β-III tubulin
TNF-α	Tumor necrosis factor-α
IL-1β	Interleukin 1β
TGIT	total gastrointestinal transit time
HBSS	Hank’s balanced salt solution
CNS	Central nervous system
DMEM	Dulbecco’s modified Eagle’s medium
PBS	Phosphate buffer saline
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulfate

Declarations

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Conflicts of interest The authors declare that they have no conflict of interest.

Availability of data and material The datasets used and/or analyzed during the current

study are available from the corresponding author on reasonable request.

Code availability GraphPad Prism Version 7.0; Image J Version 1.51J

Authors' contributions Qiang Wang and Shuang Li designed experiments and developed methodologies; Na Li, Hui Gao and Jing Xu performed the research and wrote the manuscript; Yuxin Zhang and Haiqing Chang analyzed and interpreted data; Qiang Wang, Jing Xu and Shuwen Tan revised the manuscript.

Ethics approval All procedures performed in studies involving animals were approved by the ethical standards of Institutional Animal Care and Use Committees of Xi'an Jiaotong University, Xi'an, China. Mice received humane care following the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

Consent to participate Not applicable.

Consent for publication Not applicable

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Figures

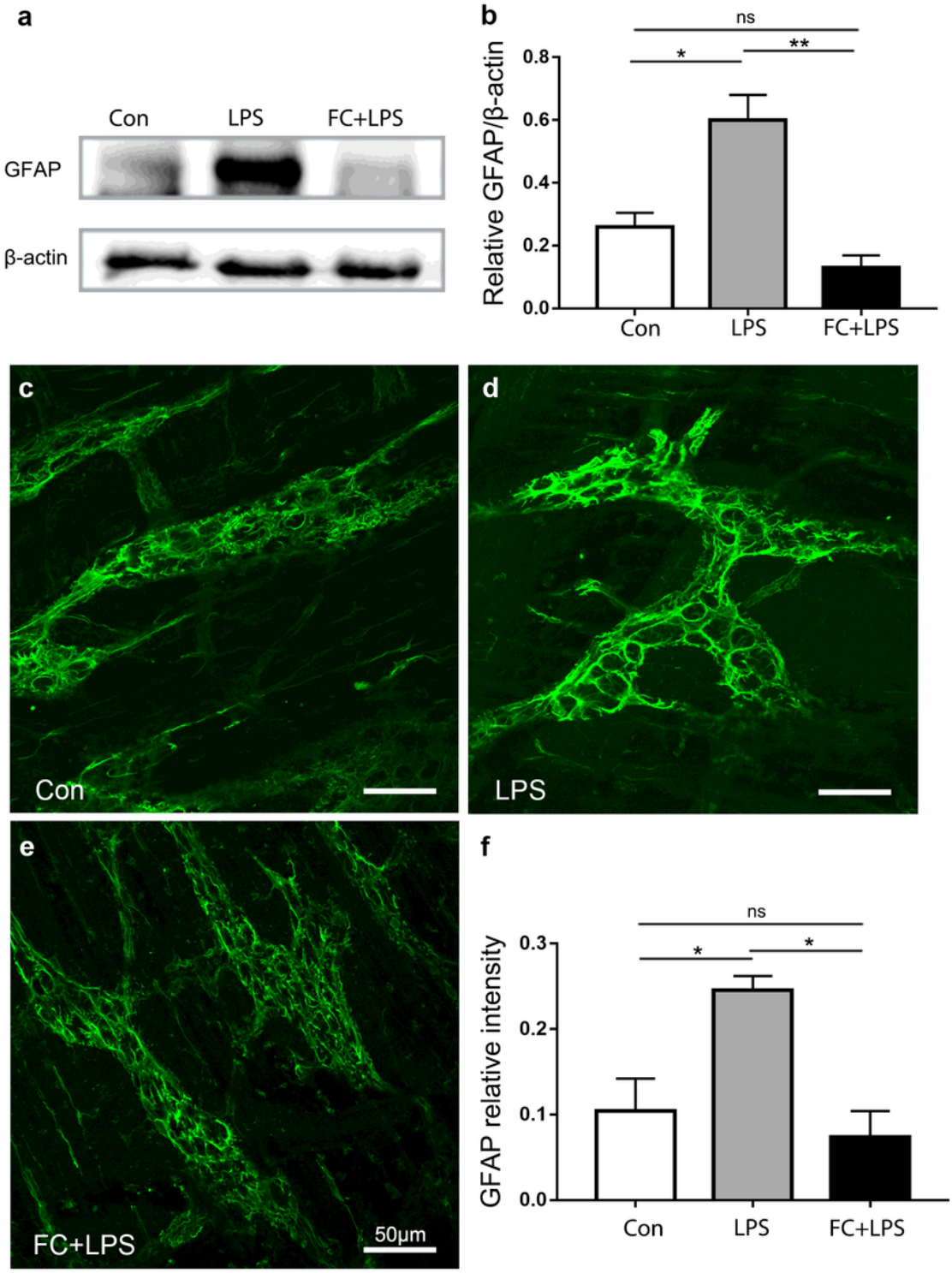

Figure 1

Effect of FC on EGCs activation in septic mice. (a-b) Western blotting showed that the protein expression of enteric glial marker GFAP was increased after the LPS injection whereas pretreatment with FC reversed the changes. (c-f) IF staining in the colon myenteric plexus showed that GFAP had the same trend among different treatment groups. One-way ANOVA, n = 6, * $P < 0.05$, ** $P < 0.01$, ns no significant difference.

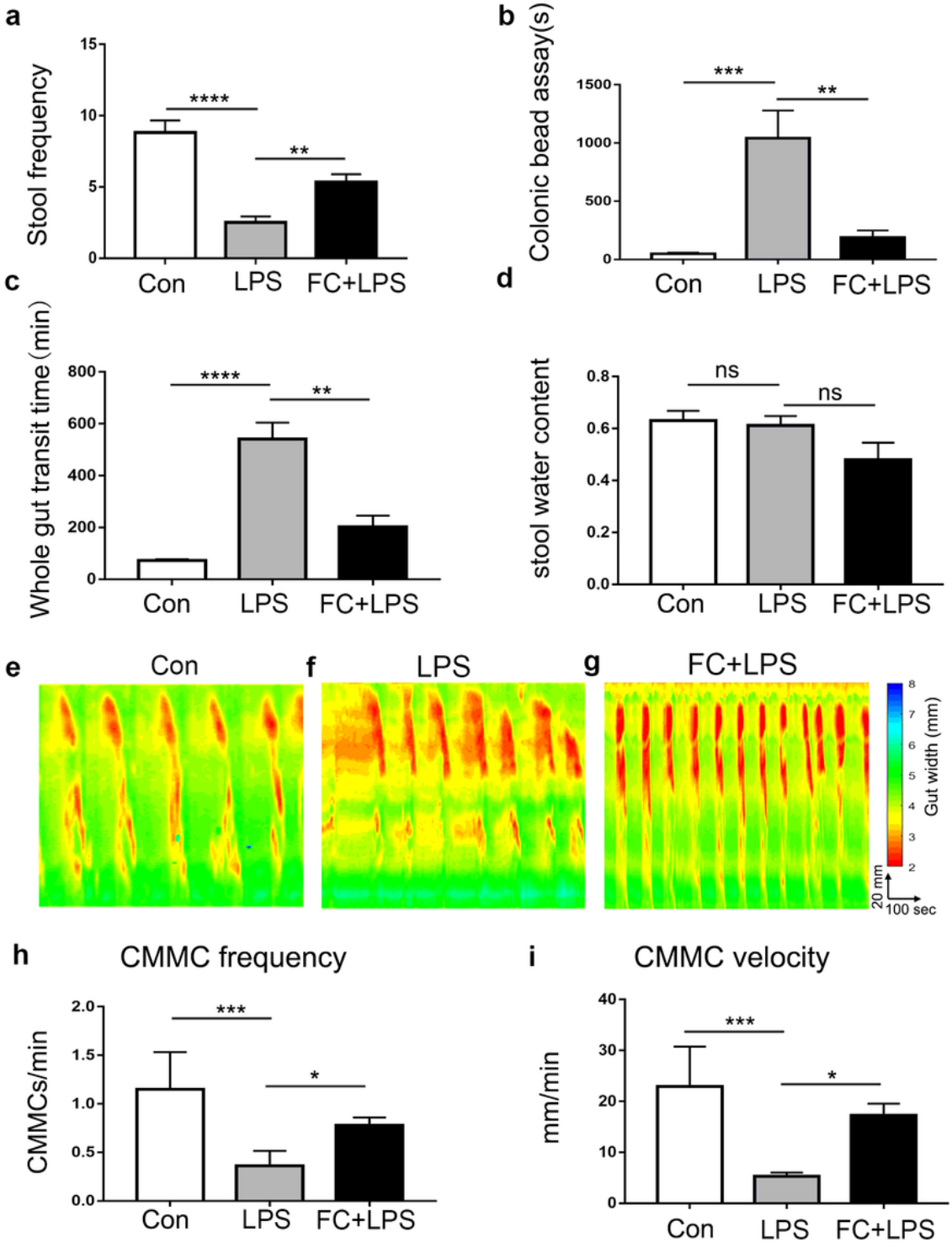

Figure 2

Effect of inhibiting reactive EGCs on intestinal motility in septic mice. The intestinal motility reduced at 48hrs after the LPS injection and partly reversed back to normal under the condition of EGC ablation. (a) Stool frequency, (b) Colonic bead assay(s), (c) Whole gut transit time, (d) Stool water content. (e-g) Spatiotemporal maps illustrating CMMCs recorded for control, LPS injection, and FC treatment. (h-j) LPS reduced CMMC frequency and velocity in the colon, while they can be significantly improved by FC

treatment. One-way ANOVA, n = 6, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns no significant difference.

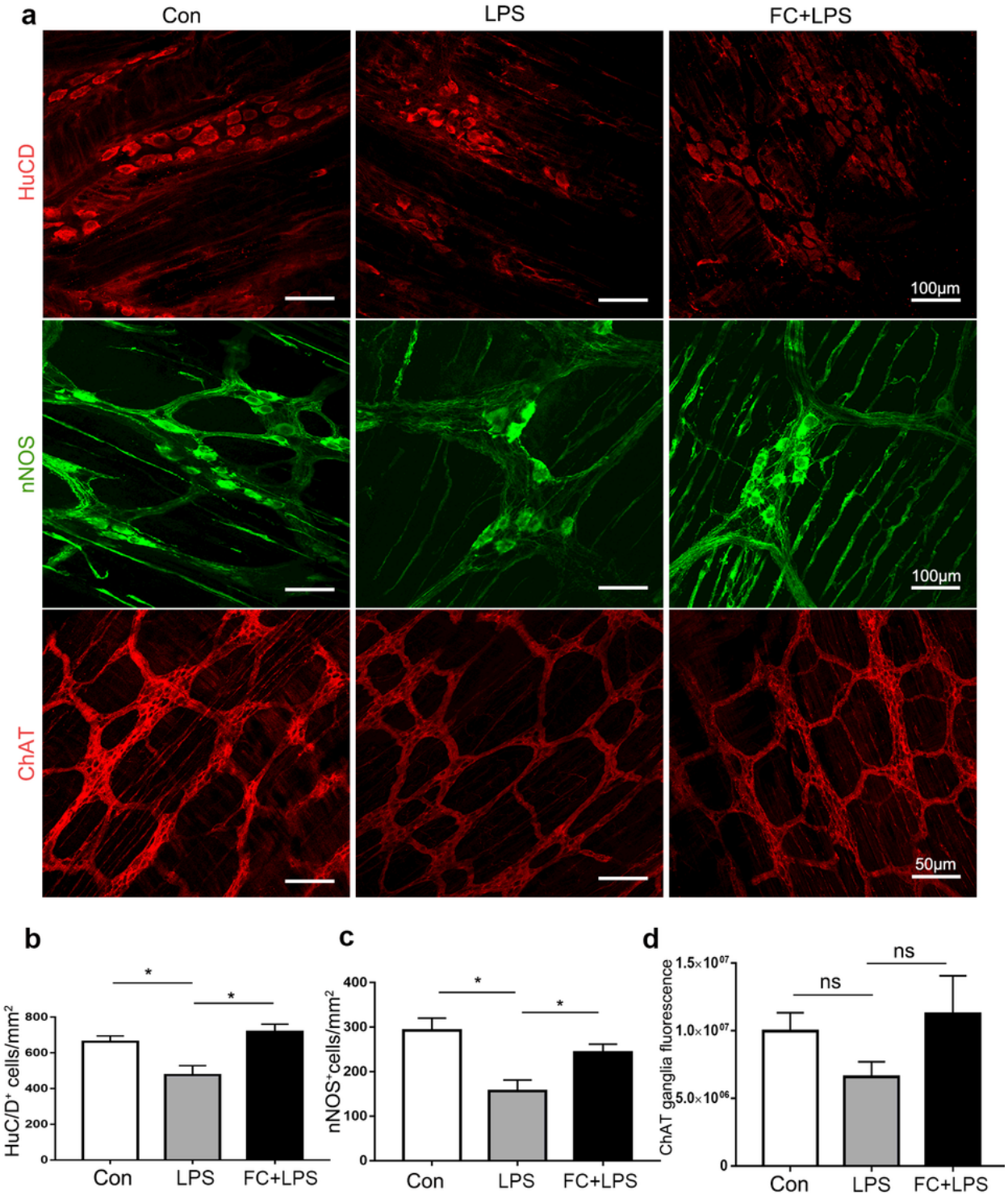

Figure 3

Effect of inhibiting reactive EGCs on the number of neurons in septic mice. (a) Representative images of immunofluorescence staining in the colonic myenteric plexus under different treatments. The number of the general neuronal population (HuC/D-immunoreactive) (b) and nitrergic neurons (nNOS-

immunoreactive) (c) reduced at 48hrs after the LPS injection, and restored to normal under the condition of EGC ablation. At the same time, myenteric ganglia fluorescence intensity (CTCF) of ChAT (d) showed no significant difference among these groups. One-way ANOVA, n = 6, * P < 0.05, ns no significant difference.

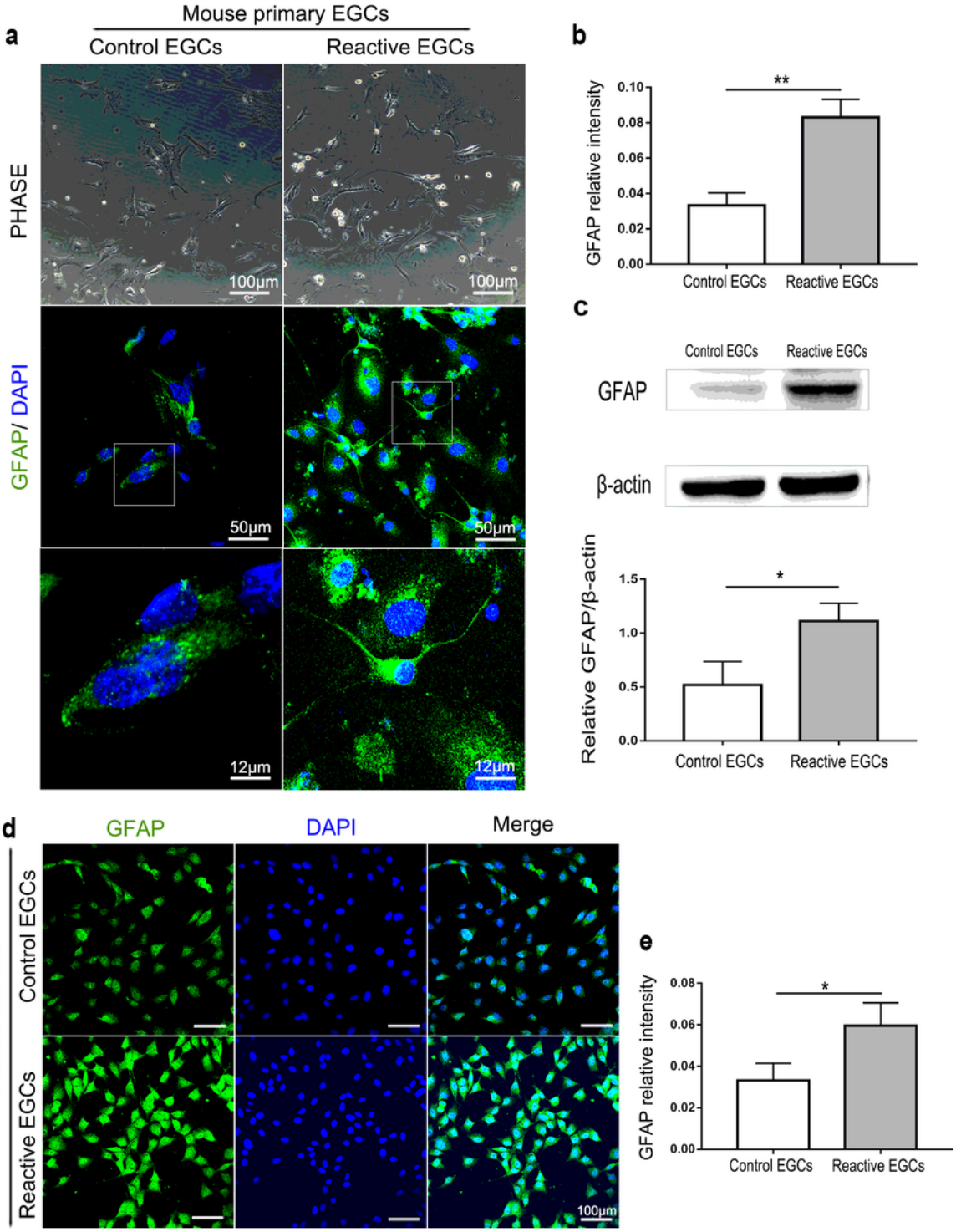

Figure 4

Reactive EGCs were induced by IL-1β and TNF-α in vitro. (a) IL-1β/TNF-α induced primary enteric glia into a reactive EGC phenotype in vitro (represent phase and fluorescent images). GFAP expression of primary enteric glia increased under IL-1β/TNF-α treatment using IHC staining (b) and Western blot (c). (d-e) IL-1β/TNF-α also induced enteric glial cell line into reactive in vitro, leading to GFAP expression raise. t-test, n = 6, * P < 0.05, ** P < 0.01.

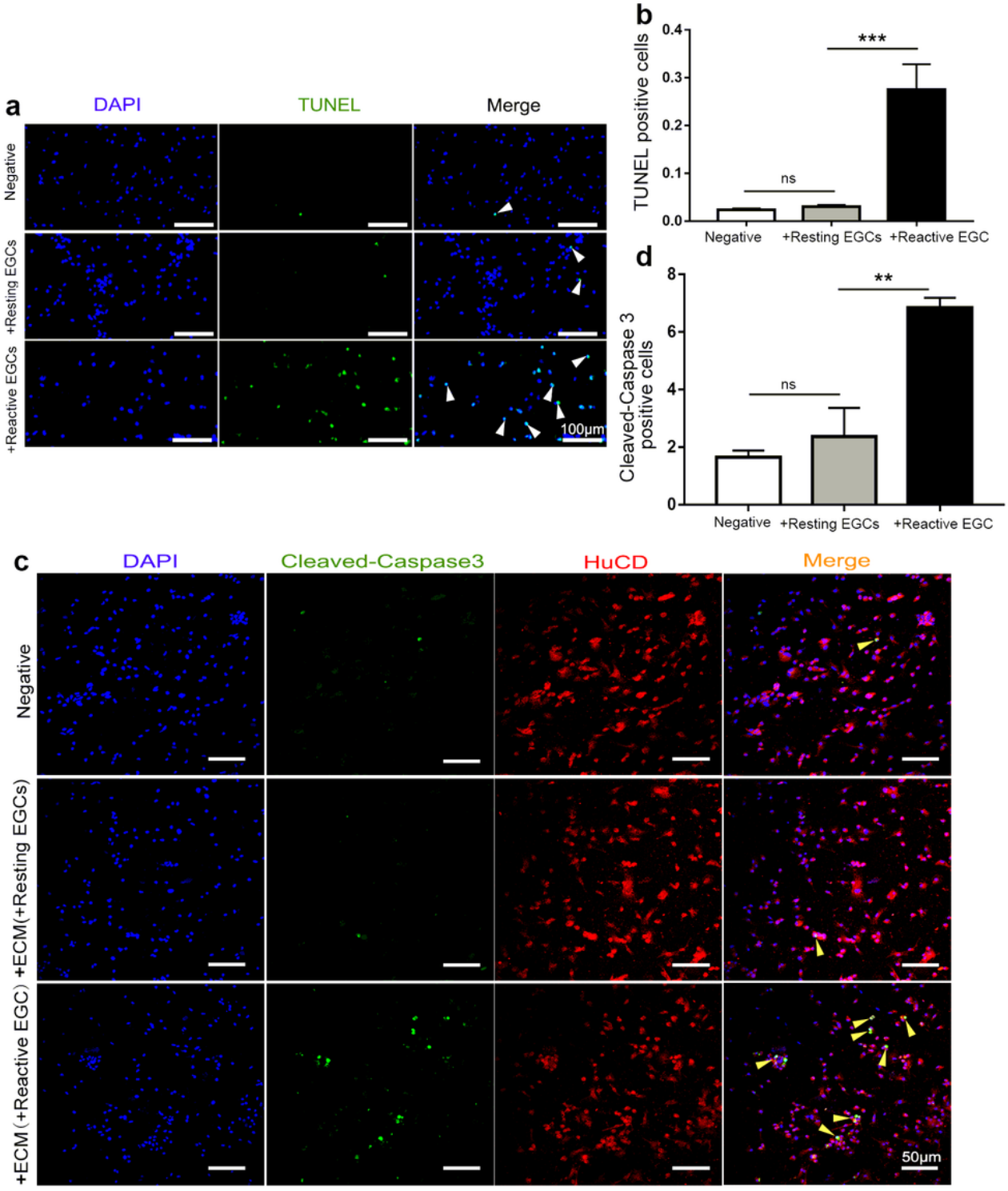

Figure 5

Effect of reactive EGCs on the apoptosis of primary enteric neurons. (a-b) TUNEL staining (the white arrows in a) showed that the apoptotic cells in the cultured enteric neurons with control, the conditional medium of normal EGCs, and reactive EGCs. The TUNEL positive cells percentage was increased at 48hrs after cultured with the conditional medium of reactive EGCs. (c-d) fluorescence staining for the cleaved form of caspase-3 in primary cultured enteric neurons (the yellow arrows in c) showed that more positive apoptotic neurons were observed in the conditional medium of the reactive EGCs group than normal EGCs. One-way ANOVA, n = 6, * P < 0.05, ** P < 0.01, *** P < 0.001, ns no significant difference.

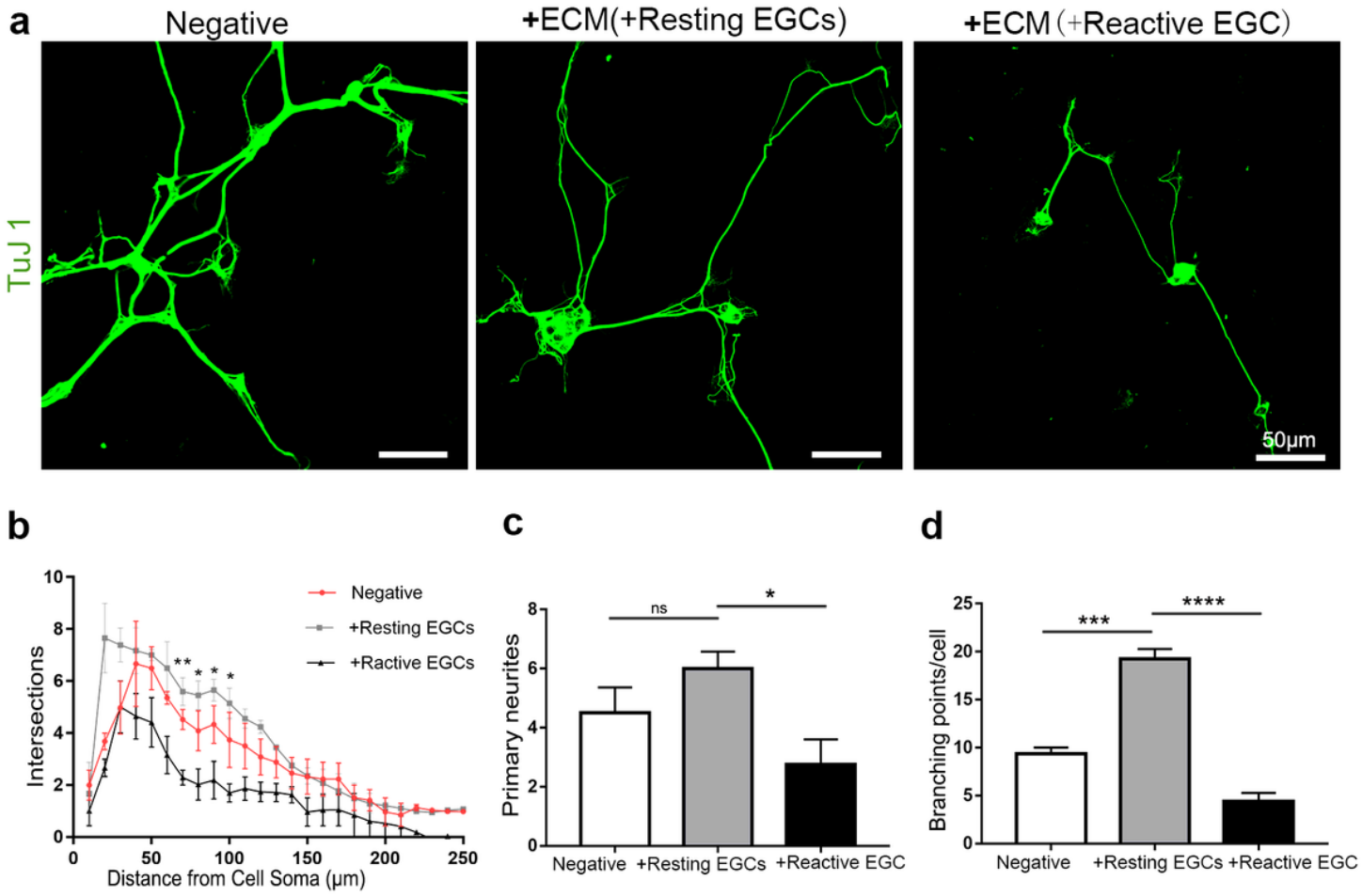

Figure 6

Effect of reactive EGCs on neurites formation in primary enteric neurons. (a) Fluorescence staining with TuJ 1 to present neuronal morphology of the cultured enteric neurons under different treatments. (b) Sholl analysis showed that the dendritic complexity of neurons cultured in the EGCs (Reactive EGCs) conditional medium was reduced. (c) The number of primary neurites and (d) branching points were decreased in the medium of the reactive EGCs group. One-way ANOVA, n = 6, * P < 0.05, *** P < 0.001, **** P < 0.0001, ns no significant difference.

Supplementary Files

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