Life Sciences 148 (2016) 254–259

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Structural changes in gastric glial cells and delayed gastric emptying as responses to early life stress and acute adulthood stress in rats

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ARTICLE INFO

Article history:
Received 11 November 2015
Received in revised form 27 January 2016
Accepted 8 February 2016
Available online 10 February 2016

Keywords:
Functional dyspepsia
Irritable bowel syndrome
Enteric glial cell
Stress
Gastric motility

ABSTRACT

Aim: Enteric glial cells (EGCs) modulate colonic motility in a maternal separation model. We aimed to investigate structural changes in gastric EGC and gastric emptying as responses to maternal separation and acute adulthood stress in rats to elucidate the pathophysiological roles of gastric EGC.
Main methods: As a chronic stress, we subjected male Wistar rats to 3 h of maternal separation during postnatal days 2–14. As an acute adulthood stress (7 weeks of age), we used the 8-h water-immersion method. We morphologically evaluated gastric EGCs using whole-mount longitudinal muscle-myenteric plexus preparations. We analyzed gastric emptying by the phenol red method.
Key finding: The area of EGC processes that apparently overlapped with neurons increased according to stress intensity (acute stress, 10.4%; maternal separation, 10.2%; maternal separation plus acute stress, 26.6%; control, 5.0%). Ratios of morphologically changed leaf-like processes to the total processes were 8.1%, acute stress; 10.3%, maternal separation; 4.0%, control. Ratio dramatically increased in the combined stress group (20.5%, $p = 0.026$ vs. control). The mean bulging head area of leaf-like processes in the combined stress group was greater by 6.4 μm^2 (control, 2.4 μm^2; $p = 0.042$). Gastric emptying in the maternal separation group was gradually delayed (104.1% at 7 weeks, 66.7% at 17 weeks, and 48.5% at 48 weeks; $p < 0.05$, respectively). Gastric emptying in the combined stress group tended to be delayed at 17 weeks (45.7% vs. 81% in controls, $p = 0.066$).
Significance: Gastric EGCs exhibited structural changes according to stress intensity, which may be associated with stress-induced dysfunction of the stomach.

1. Introduction

Functional dyspepsia (FD) and irritable bowel syndrome (IBS) are functional gastrointestinal disorders (FGIDs). FD pathogenesis involves multiple factors including visceral hypersensitivity and gastroduodenal motility disorders based on the results of genetic, environmental, psychological, and physiological studies [1–3]. Moreover, brain–gut interactions may be of particular importance in the pathogenesis of FGIDs, because physiological functions of the gastroduodenal tract are generally achieved by signal transmission from the central nervous system (CNS) to the enteric nervous system (ENS), mediated by the autonomic nervous system (ANS) [4], and this interaction is modulated by systemic stress via the hypothalamus–pituitary–adrenal (HPA) axis [5]. Therefore, FD pathogenesis is proposed to involve functional abnormalities throughout the CNS and the gastroduodenal tract [6–9].

Clinical surveillance has shown a high prevalence of childhood trauma such as sexual or physical abuse in FD patients [10,11], and abdominal symptoms are more severe in FD patients with a history of abuse than in those without [12]. Childhood trauma is also associated with gastric hypersensitivity, gastric motor dysfunction, or differences in brain activity in FD patients [11,13,14]. Furthermore, secretion of adrenocorticotropic hormone and cortisol with stress exposure is higher in healthy adult subjects with a history of childhood trauma compared to those without [15]. Thus, childhood trauma may result in a more sensitive response of the HPA axis, motor dysfunction of the gastrointestinal tract, and dyspeptic symptoms in adult FD patients. Accordingly, pathological changes in the gastric wall including the ENS may exist in FD patients, since there are no macroscopic changes of the gastric mucosa. Therefore, microscopic cellular changes, especially of the ENS, should be elucidated using a stress model, since an animal model of idiopathic FD has not been definitively established.

The maternal separation model (a well-established experimental model of early-life stress) along with additional acute stress in adulthood causes colonic hyper-motility, barrier dysfunction, and visceral hypersensitivity via various neurotransmitters [16–18]. Our previous

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http://dx.doi.org/10.1016/j.lfs.2016.02.025
0024-3205/

report showed that colonic enteric glial cells (EGCs) were structurally altered (with elongation and/or bulbous terminal swelling of processes) with external stress stimulation. EGCs are associated with stress-induced colonic hyper-contraction in rats with the combination of maternal separation and acute stress [19]. EGCs together with enteric neurons are components of the ENS in the submucosal and myenteric plexuses. Recent studies have also shown that EGCs contribute to mucosal barrier function, intestinal inflammation, enteric neurotransmission, gastrointestinal motility, neuronal protection, and neurogenesis [20–22]. These findings suggest that EGCs may play an important role in the regulation of gastrointestinal function via the ENS rather than simply providing a supportive framework for neurons according to various pathophysiological conditions.

However, it is still unknown whether changes in gastric EGCs and related gastric dysmotility occur in response to stress. Considering the pathogenesis of FD, maternal separation with an additional acute-stress model may be suitable for evaluating the pathogenesis of FD. Therefore, we investigated structural changes in gastric EGCs and gastric emptying in maternally separated rats using an additional acute-stress model.

2. Materials and methods

2.1. Animals

Primiparous timed-pregnant female Wistar rats (Charles River Japan, Inc., Yokohama, Japan, gestational day 14) were allowed free access to water and food and were maintained at 22–24 °C with controlled lighting (light from 0800 to 2000 daily). The animal ethics committee of Osaka City University approved all experiments.

2.2. Maternal separation

Maternal separation was performed daily for 3 consecutive hours (0900–1200) on postnatal days 2–14 [23]. Briefly, pups were separated from their dams and placed in individual cages on heated pads (28–30 °C) in another room. Control pups were left with their dams. On postnatal day 22, all pups were weaned. To avoid hormonal cycle-induced variations, only male pups were analyzed.

2.3. Acute stress

We used a rat model of fatigue with continuous stressful restraint as an acute stressor according to previous studies [24]. Briefly, rats 7 weeks of age were housed for 8 h (0800–1600) in cages filled with water (23 ± 1 °C) to a height of 2.0 cm.

2.4. Experimental protocol

Maternal separation was used as the infantile chronic stressor, and water-immersion stress was used as the acute stressor in adulthood. We divided the rats into 4 groups: 1) control (no stress); 2) acute stress in adulthood alone; 3) maternal separation prior to weaning alone; and 4) maternal separation prior to weaning plus acute stress in adulthood (combined stress). All rats were sacrificed by cervical dislocation at 7 weeks of age or 24 h after acute stress, and gastric tissue samples were obtained.

2.5. Immunofluorescent staining

The rat stomachs were opened along the greater curvature, pinned flat with the mucosal side up, and fixed with acetone (4 °C, 1 h). According to the method previously published [19], whole-mount longitudinal muscle-myenteric plexus (LMMP) tissues were prepared. After blocking with 5% normal donkey serum in phosphate-buffered saline containing 0.3% Triton X-100 (4 °C, 1 h), each LMMP preparation was incubated overnight at 4 °C with primary antibodies. Rabbit anti-glial fibrillary acid protein (GFAP) (a marker of glial cell cytoplasm, 2 μg/mL, Abcam) and mouse anti-HuC/D (a marker of enteric neurons, 0.4 μg/mL, Molecular Probes) were used as primary antibodies. After washing, tissues were incubated with secondary antibodies (donkey anti-rabbit Alexa Fluor 488, 4 μg/mL, and goat anti-mouse Alexa Fluor 633, 4 μg/mL, Molecular Probes) for 2 h at room temperature. Preparations were visualized with confocal microscopy (LAS AF, TCS SP5, Leica Microsystems) with a 63× oil-immersion objective lens (numerical aperture 1.4). Z-series of scans (0.5 μm) and single 512 × 512 pixel images (0.76 μm/pixel) were captured.

2.6. Quantitative analysis of areas with glial processes that apparently overlap with neurons and bulging head area of leaf-like processes

We measured areas of GFAP-positive hyperplasia of glial processes that apparently overlapped with neurons using the National Institutes of Health Image-J software (version 1.44) as previously reported [19]. A mean ratio of the total area of EGC processes to the whole area of HuC/D-positive neurons was evaluated in the fixed threshold condition. In a blinded manner, we (YF and KT) counted EGC processes that apparently overlapped with each neuronal cell body per ganglion and examined the structure of the EGC processes. Image analyses were performed for 30 neurons in 4 ganglia that were randomly selected from each rat in each group ($n = 4$). After confirming the terminal glial processes, the glial processes were classified according to their terminal structure as either filamentous (no obvious neck or bulbous terminal swelling) or leaf-like (a neck region with bulbous terminal swelling). We calculated the mean ratio of leaf-like processes to total processes per ganglion and the mean bulging head area of leaf-like processes in the 4 groups at 7 weeks. Bulging head area of leaf-like processes was calculated according to the measurement formula for an oval area: bulging head area = π × width of bulging head × length of bulging head.

2.7. Gastric emptying

For rats at 7, 17, and 48 weeks of age, gastric emptying was measured according to the previously reported method using 1 mL of phenol red (100 μg/mL) [24]. In brief, 1 mL of phenol red (100 μg/mL) was orally injected into the stomach of each rat. Each rat was sacrificed 15 min after phenol red administration and the stomach was removed immediately. S1 and S2 solutions were collected according to the method previously reported [24]. Each absorbance was measured at a wavelength of 570 nm using a microplate reader (MTP-500; Corona Electric, Ibaragi, Japan). Gastric emptying was calculated in each rat and expressed as a ratio to that in the 7-week control group (100%).

2.8. Statistical analysis

Data were expressed as means ± standard error. We statistically analyzed the area ratio of glial process overlapped neurons, the ratio of leaf-like processes to total processes per ganglion, and gastric emptying among multiple groups using analysis of variance with Bonferroni's correction. The significance level was set at $p < 0.05$.

3. Results

3.1. Histological evaluation of gastric EGCs

Most EGC (GFAP-positive) processes were present around HuC/D-positive neurons in the two-dimensional projections. In the control group, a small number of EGC processes that apparently overlapped with neurons were observed. However, the density of the EGC processes that apparently overlapped with neurons progressively increased according to the type of stress (Fig. 1A). The two-dimensional area of apparent overlap of EGC processes with neurons was semi-quantitatively