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Cholecystokinin-8 increases Fos-like immunoreactivity in the brainstem and myenteric neurons of rats through CCK_1 receptors

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Abstract

To examine the role of cholecystokinin$_1$ receptor (CCK_1) in the activation of brainstem and myenteric neurons by CCK, we compared the ability of exogenous CCK-8 to induce Fos-like immunoreactivity (Fos-LI) in these neurons in Otsuka Long–Evans Tokushima Fatty (OLETF) rats, lacking CCK_1 receptors, and Long–Evans Tokushima Otsuka (LETO) controls. Five groups ($n = 4$ rats per group) of OLETF rats, and five LETO control groups, were injected intraperitoneally (IP) with 5, 10, 20, and 40 μg/kg CCK-8 or saline. Forty-micrometer brainstem sections containing the area postrema, nucleus of the solitary tract, and the dorsal motor nucleus of the vagus, and myenteric neurons of the duodenum, jejunum, and ileum underwent a diaminobenzidine reaction enhanced with nickel to reveal Fos-LI. CCK-8 did not increase Fos-LI in any of the tested neurons in the OLETF rats. CCK-8 increased Fos-LI in the brainstem of the LETO rats in a dose dependent manner. In the LETO rats only 40 μg/kg CCK-8 increased Fos-LI in the myenteric plexus of the jejunum. This study demonstrates that CCK-8 activates the brainstem and myenteric neurons through the CCK_1 receptor.

Keywords: Brainstem; Hindbrain; Enteric nervous system; Cholecystokinin; CCK_1 receptor ;OLETF; LETO; Fos; Myenteric plexus; Rat

1. Introduction

In 1984, inbreeding of a spontaneously diabetic Long–Evans rat suffering from polyuria, polydipsia, and obesity produced a colony of rats known as Otsuka Long–Evans Tokushima Fatty (OLETF). The control line derived from the same original colony, known as Long–Evans Tokushima Otsuka (LETO), fail to develop the previous clinical signs even after more than 20 generations of inbreeding. The OLETF rats are characterized by increased rates of weight gain beginning at 5 weeks of age, hyperglycemia, and non-insulin dependent diabetes mellitus, which starts at the age of 18 weeks, and insulin deficiency after the age of 65 weeks [6]. Studies examining pancreatic function in OLETF rats demonstrated that these rats failed to secrete amylase in response to exogenous cholecystokinin-8 (CCK-8). The previous finding led to an evaluation of the CCK receptor genes in these rats, which demonstrated that OLETF rats had a 6.8-kb deletion in the gene for the cholecystokinin$_1$ (CCK_1) receptor that spanned the promoter region and the first and second exon. This mutation results in a failure to produce a functional CCK_1 receptor [20]. On the other hand, CCK_2 receptors are not affected by this mutation [2].

Cholecystokinin is a hormone secreted by the I cells of the small intestine and neurons in the central and enteric nervous systems (reviewed recently in [13]). It evokes numerous digestive functions such as gallbladder and smooth muscle contraction, stimulation of pancreatic exocrine secretion, reduction of food intake, and inhibition of gastric emptying. The actions of CCK are mediated through interactions with two G-protein coupled receptors, CCK_1 and CCK_2. The distribution of these receptors includes the gastrointestinal (GI) tract, pancreas, gallbladder, and central and peripheral nervous systems including vagal afferents.

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The absence of the CCK_1 receptor in OLETF rats makes them a suitable model for studying the various functions of CCK. In Sprague–Dawley rats, CCK-8 increases Fos-like immunoreactivity (Fos-LI), a neuronal activation marker [10], in the myenteric neurons of the small intestine and in the brainstem [1,11,12,14–18]. Although antagonist data have suggested that the majority of this activation is mediated through the CCK_1 [14], and not CCK_2 [21], receptor, the OLETF rat provides the opportunity for critically assessing this assertion and potentially distinguishing differential mediation at brain and intestinal sites.

2. Materials and methods

2.1. Animals

Twenty male OLETF (average 230 g) and 20 male LETO rats (average 230 g), 6–10 weeks of age were housed singly in wire-mesh cages, in a controlled environment (lights were on from 06:00 to 18:00 h and temperature was maintained at 21.5 °C). Rats had ad libitum access to water and pelleted rodent chow (Teklad, WI). To enhance adaptation to the laboratory, we handled each rat for 10 min/day for the first 7 days.

2.2. Experimental procedures

The OLETF rats were assigned to five groups ($n = 4$ rats per group). Rats were deprived of food, but not water, beginning at 6:00 p.m. on the day prior to an experiment. At 8:00 a.m., rats received intraperitoneally (IP) injections of 0.5 ml saline or 1 of 4 doses of CCK-8 (Bachem, CA) 5, 10, 20, and 40 μg/kg. The LETO rats were also divided into five groups ($n = 4$ per group) and received the same treatments as the OLETF rats.

Ninety minutes after the IP injection, rats were anesthetized with sodium pentobarbital (10 mg/kg, IP) and perfused transcardially in two stages. First, the rats were perfused with 500 ml of Krebs solution (Krebs saline formula: 119 mM NaCl, 4.7 mM KCl, 1.2 mM $NaH_2PO_4 \cdot H_2O$, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11.1 mM glucose, and 1 M $CaCl_2$) to collect the small intestine, and second with 500 ml of 4% formaldehyde (made in 0.1 M phosphate-buffered saline (PBS)) to collect the brainstems. The small intestine was exposed through a midline abdominal incision and the duodenum, jejunum, and ileum were identified. On the basis of our previous experiments [11–18], the duodenal sample was 5–10 cm aborad from the pylorus, the jejunal sample was 20–25 cm aborad from the pylorus, and the ileum sample was 5 cm orad from the ileo–cecal junction. The lumens of the removed segments were rinsed with Krebs solution. The segments were placed in Sylgard-coated Petri dishes (World Precision Instruments, FL), opened along the mesenteric attachment, stretched, pinned with the mucosal side up, and stored overnight in Zamboni's fixative at 4 °C.

On the next day, the tissues were unpinned and cleared three times in 100% dimethyl sulfoxide (DMSO), 10 min each time, followed by three 10 min rinses with 0.1 M PBS, pH 7.4. Wholemounts (approximately, 1 cm^2) of longitudinal smooth muscle with adhering myenteric plexus from the duodenum, jejunum, and ileum were prepared using a dissecting microscope and processed for immunohistochemical detection of Fos-LI.

After collection, the brainstems were postfixed with 4% formaldehyde for 2 h, and placed in 25% sucrose overnight at room temperature. The brainstems were sectioned at 40 μm on a cryostat at −20 °C. Consistent with previous work done in Sprague–Dawley rats, the areas cut included the following levels according to the Paxinos and Watson rat brain atlas [8]: the area postrema (AP, −4.5 mm caudal to interaural plane), nucleus of the solitary tract (NTS, −4.5 and −4.8 mm caudal to interaural plane), and the dorsal motor nucleus of the vagus (DMV, −4.5 and −4.8 mm caudal to interaural plane). Sections were taken at multiple levels of the NTS to insure sampling from sites receiving both gastric and intestinal vagal afferent innervation.

2.3. Immunohistochemistry

Based on our previous methods [11–18], the wholemount preparations and brainstem sections were incubated for 24 h at room temperature in primary antiserum raised in rabbit against a peptide representing amino acids 4–17 of human Fos (Oncogene, Ab-5, San Diego, CA [1:12,000 dilution]). After a subsequent overnight incubation in biotintylated donkey anti-rabbit serum (Jackson ImmunoResearch Laboratories, PA [1:500 dilution]) the tissues were incubated for 3 h in avidin conjugated to horseradish peroxidase (HRP) then washed with 0.01 M Tris PBS and processed to reveal HRP activity using diaminobenzidine (DAB, Sigma, MO) intensified with nickel.

2.4. Counting procedure

Two observers, blinded to the treatments, counted fos-positive cells from all of the sections. The fos-positive cells at each intestinal level (duodenum, jejunum, and ileum) of every animal were counted. The final count for each intestinal level represents the average from 10 non-overlapping, 40× microscopic fields. Automated computer software (ImagePro Plus, Media Cybernetics) was utilized to count Fos-LI in the brainstem sections. Fos-positive cells within the AP, NTS, and DMV were counted at the −4.5 mm caudal to interaural plane, and within the NTS and DMV were counted at the −4.8 mm caudal to interaural plane according to the rat brain atlas [8].

2.5. Statistical analysis

To analyze the effect of the different CCK-8 doses on intestinal and brainstem levels in the OLETF and the LETO rats,