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Research Report

Strain differences in myenteric neuron number and CCK_1 receptor mRNA expression may account for differences in CCK induced c-Fos activation

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Abstract

We utilized a diaminobenzidine reaction enhanced with nickel to compare dorsal vagal complex (DVC) and myenteric neuronal Fos-Like immunoreactivity (Fos-LI), in response to sulfated cholecystokinin-8 (CCK-8) (5, 10, 20, 40 μg/kg), among Sprague–Dawley (SD), Standard Long–Evans (SLE), Otsuka Long-Evans Tokushima Fatty (OLETF), and Long–Evans Tokushima Otsuka (LETO) rats. All rat strains but OLETF expressed Fos-LI in response to CCK-8. In addition, SD rats expressed more Fos-LI in the area postrema and myenteric neurons than SLE and LETO rats. To investigate the basis for these differences, we utilized cuprolinic blue staining, which stains neuronal cell bodies, to quantify the number of myenteric neurons, and a reverse transcriptase chain polymerase reaction to measure the gene expression of CCK_1 receptor in the gut. We found that SD rats have significantly more duodenal myenteric neurons than the other strains. In addition, this strain expressed significantly higher levels of the CCK_1 gene in both the duodenum and jejunum than the other strains. In conclusion, SD rats may express more myenteric Fos-LI in response to CCK due to increased numbers of myenteric neurons or more intestinal CCK_1 receptors than the other strains of rats.

Theme: Endocrine and autonomic regulation
Topic: Neuroendocrine regulation

Keywords: Sprague–Dawley; Long–Evans; LETO; OLETF; Fos; Myenteric plexus; Dorsal vagal complex; Cholecystokinin; CCK_1 receptor; NTS; Area postrema; Immunohistochemistry

1. Introduction

Cholecystokinin (CCK) (reviewed recently in [14]) is a hormone secreted by the I cells of the gastrointestinal (GI) tract, and neurons in the central and enteric nervous systems (CNS and ENS, respectively). CCK plays a major role in the digestive process, evoking functions such as gallbladder contraction, the inhibition of gastric emptying, the stimulation of pancreatic secretion, and satiety. The actions of CCK are mediated through its interactions with two G-protein coupled receptors, CCK_1 and CCK_2. These receptors are widely distributed throughout the GI tract and central and peripheral neurons.

Recently, a line of rats lacking the CCK_1 receptor has been identified. From a spontaneous occurrence of diabetes and obesity in an outbred colony of Long–Evans rats, a line of rats that is characterized by diabetes, polyuria, polydipsia, and obesity was produced by selective mating. These rats are known as Otsuka Long–Evans Tokushima Fatty (OLETF) rats. These rats show increased rates of weight gain, hyperglycemia, and non-insulin dependent diabetes mellitus [5]. A control strain of rats, known as Long–Evans Tokushima Otsuka (LETO) does not show any of the clinical signs discussed above and these have been considered normal Long–Evans rats.

In characterizing the pancreatic function of OLETF rats, it was noted that they did not respond to exogenous CCK [1]. This led to the demonstration that these rats lack CCK_1 receptors because of a 6.8 kb deletion in their CCK_1 receptor

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gene, a deletion that spans the promoter region and the first and second exons. This deletion results in the absence of a functional CCK_1 receptor. Due to this mutation, the OLETF rats provide a useful model for studying the various functions of CCK.

To demonstrate a possible role for the ENS in physiological responses evoked by CCK-8, we provided the following data [2,3,8–15,17,18]. Exogenous CCK-8 and endogenous CCK, secreted in response to intestinal nutrient infusion of oleate, increased Fos-Like immunoreactivity (Fos-LI), a marker for neuronal activation [7], in the myenteric neurons of the rat small intestine and the dorsal vagal complex (DVC), which also receive afferent input from the vagus nerve, the main ex-trinsic innervation of the gastrointestinal (GI) tract. Increased DVC, but not myenteric, Fos-LI, was vagaly dependent.

Furthermore, in studies characterizing the CCK receptor subtype underlying the ability of CCK to activate hindbrain and enteric neurons, we demonstrated what appeared to be a strain difference in the responsiveness of enteric neurons to CCK. Utilizing Fos-LI as a marker of neuronal activation, we found that although CCK potently induced c-Fos expression in the enteric nervous system of Sprague–Dawley rats, 40 μg/kg, a supraphysiological dose of CCK, was the only dose that increased Fos-LI in the LETO rats. Since there were a number of differences in the design of the studies, we could not be sure whether the difference was age related (6–9 weeks LETO rats may not have a fully functional CCK_1 receptor in their myenteric neurons) or whether there was something specific about the LETO control strain in the distribution of their CCK_1 receptors. The current study was designed to investigate the reasons for this apparent strain difference. We compared Fos-LI in the myenteric and brainstem neurons in response to CCK-8 in four strains of 14–20 week old rats—Sprague–Dawley (SD), LETO, OLETF, and Standard Long–Evans rats (SLE).

2. Materials and methods

2.1. Animals

We used 47 male rats from each strain. Weights ranged between 280 and 380 g. Rats were housed singly in wire-mesh cages, in a controlled environment (lights were on from 0600 to 1800 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

Rats from each of the tested strains were assigned to one of 5 treatment groups: saline ($n = 5$), 5 ($n = 9$), 10 ($n = 8$), 20 ($n = 16$), and 40 ($n = 9$) μg/kg CCK-8, and deprived of food, but not water, beginning at 6:00 PM on the day prior to an experiment. At 8:00 AM, the rats received an IP injection of 0.5 ml saline or 1 of 4 doses of CCK-8 (μg/kg, Bachem, CA): 5, 10, 20, 40.

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 in mM: 119 NaCl, 4.7 KCl, 1.2 $NaH_2PO_4 \cdot H_2O$, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 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 and jejunum were collected. On the basis of our previous experiments [2,3,8–15,17,18], the duodenal sample was 5–10 cm aborad from the pylorus and the jejunal sample was 20–25 cm aborad from the pylorus. The lumens of the removed segments were rinsed with Krebs solution, and the segments were placed in Sylgard-coated Petri dishes (World Precision Instruments, FL), opened along the mesenteric attachment, stretched and 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. Whole mounts (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 [6]: 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 [3,8–15,18], the whole mount 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 Immuno-Research Laboratories, PA [1:500 dilution]), the tissues