Low-affinity CCK-A receptors are coexpressed with leptin receptors in rat nodose ganglia: implications for leptin as a regulator of short-term satiety

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**Abstract**

The paradigm for the control of feeding behavior has changed significantly. Research has shown that leptin, in the presence of CCK, may mediate the control of short-term food intake. This interaction between CCK and leptin occurs at the vagus nerve. In the present study, we aimed to characterize the interaction between CCK and leptin in the vagal primary afferent neurons. Single neuronal discharges of vagal primary afferent neurons innervating the gastrointestinal tract were recorded from rat nodose ganglia. Three groups of nodose ganglia neurons were identified: group 1 responded to CCK-8 but not leptin; group 2 responded to leptin but not CCK-8; group 3 responded to high-dose CCK-8 and leptin. In fact, the neurons in group 3 showed CCK-8 and leptin potentiation, and they responded to gastric distention. To identify the CCK-A receptor (CCKAR) affinity states that colocalize with the leptin receptor OB-Rb, we used CCK-JMV-180, a high-affinity CCKAR agonist and low-affinity CCKAR antagonist. Administration of CCK-JMV-180 eliminated the synergistic interaction between CCK-8 and leptin. As expected, immunohistochemical studies showed that CCK-8 administration significantly potentiated the increase in the number of c-Fos–positive neurons stimulated by leptin in vagal nodose ganglia. We conclude that both low- and high-affinity CCKAR are expressed in nodose ganglia. Many nodose neurons bearing low-affinity CCKAR express OB-Rb. These neurons also respond to mechanical distention. An interaction between CCKAR and OB-Rb in these neurons likely facilitates leptin mediation of short-term satiety.

**Keywords:** vagal afferents, potentiation between CCK and leptin, CCK-JMV-180
Introduction

The presence of CCK receptors in the rat vagus nerve has been confirmed using in vitro receptor autoradiography (40). The vagal CCK receptors were determined to be predominantly type A (23), as the CCK-A receptor antagonist L-364,718 completely abolished $^{125}$I-CCK binding and nonsulfated CCK had no effect. Electrophysiological studies in rats and ferrets provided functional evidence of CCK stimulation of vagal afferent pathways (3,17). Li et al. (17) recorded the unitary activities of sensory vagal neurons using microelectrodes implanted in rat nodose ganglia. CCK infusion at 40 pmol/kg per hour, which mimics postprandial levels, evoked a marked increase in neuronal discharge over basal (17). Similar studies in ferrets (3) showed that mucosal vagal afferent fibers from the duodenum are highly sensitive to CCK-8.

Similar to CCK-A receptors in rat pancreatic acini, vagal CCK receptors exist in both low- and high-affinity states (18). Currently, it is not known whether the CCK receptors at these two sites (i.e., pancreatic acini and vagus nerve) represent distinct proteins or different affinity states of the same receptor protein. Vagal high-affinity CCK receptors appear to be important in the mediation of postprandial pancreatic enzyme secretion (11), whereas low-affinity CCK receptors are involved in regulating short-term satiety (38). Using a rat model with a pancreatic–biliary cannula, Li et al. studied the effects of the CCK analogue CCK-JMV-180 (JMV-180) on exocrine pancreatic secretion (11). JMV-180, which acts as an agonist on high-affinity CCK receptors and an antagonist on low-affinity CCK receptors, was used to identify the vagal CCK affinity states involved in the mediation of the vagal afferent response to endogenously released CCK evoked by the diversion of bile–pancreatic juice in rats (18). JMV-180 failed to block the pancreatic response to physiological doses of CCK-8. Further, it enhanced, rather than inhibited, pancreatic protein secretion in response to intraduodenal administration of 18% casein, which
had been shown to release endogenous CCK. These observations indicate that both exogenous and endogenous CCK evoke pancreatic secretion by acting on high-affinity CCK receptors. On the other hand, Wheatherford et al. (38) demonstrated that JMV-180 dose-dependently reversed the effect of CCK-8 on satiety, suggesting that the anorexic activity of CCK is mediated through an interaction with the low-affinity CCK receptor site. Hence, it appears that different affinity states of the vagal CCK-A receptors (CCKAR) mediate different digestive functions.

The long form of the leptin receptor (OB-Rb) has been found in a subpopulation of vagal afferent neurons (6,7,28). Extracellular recording of vagal afferent fibers has revealed that exogenous leptin alters the firing rate of these fibers and, further, there may be a cooperative activation of these fibers by CCK and leptin (37). Functional studies have shown that leptin interacts with CCK to regulate food intake and body mass. For example, leptin-induced reduction of body weight was enhanced when CCK was coadministered with leptin (20). In addition, administration of leptin facilitated CCK-induced reduction of meal size (1) and total daily caloric intake (21). These studies clearly indicate that CCK interacts with leptin at the level of the vagal afferent neurons to control food intake and body weight. It is conceivable that CCK and leptin may act separately on the nodose ganglia to regulate pancreatic secretion and body weight, respectively; and further, they may act synergistically to stimulate a specific population of vagal primary afferent neurons to regulate short-term satiety. In the current investigation, we performed electrophysiological and immunohistochemical studies in rats to determine the distribution pattern of high- and low-affinity CCKAR and leptin receptors on the vagal nodose neurons. We found that both low- and high-affinity CCKAR are expressed in different nodose ganglia neurons. Many of the nodose neurons bearing low-affinity CCKAR also expressed OB-
Rb. These neurons also responded to mechanical distention. An interaction between CCKAR and OB-Rb in the nodose neurons likely facilitates the leptin mediation of short-term satiety.

**Materials and Methods**

**Materials**

CCK-8 and leptin were purchased from Research Biochemical International (Natick, MA). JMV-180 was purchased from Research Plus (Manasquan, NJ). CCKAR goat polyclonal antibody (sc-16172) and mouse anti–Ob-R (B-3) monoclonal antibody (sc-8391) were purchased from Santa Cruz Biotechnology (Santa Cruz CA). c-Fos rabbit polyclonal antibody Ab-5 was purchased from Calbiochem (LaJolla, CA).

**Animal Preparation**

Experiments were performed on adult male Sprague Dawley rats weighing 270–350 g that had been given free access to food and water. The animals were anesthetized with a mixture of xylazine and ketamine (13 and 87 mg/kg body weight, respectively). Supplemental doses of the anesthetic agents were administered as needed to maintain a deep level of anesthesia and muscle relaxation. To prevent any undesirable movements, paralysis was induced with pancuronium bromide (0.1 mg/kg, intravenous; supplemented as required). The animals were ventilated with a respirator – a tracheal tube permitted artificial ventilation with room air (75–85 strokes/min, 3.5–4.0 cm$^3$ tidal volume). A midline abdominal incision exposed the abdominal vagus, the stomach, and the duodenum. We stimulated the subdiaphragmatic vagus nerve by placing a pair of Teflon-coated, pure gold wire electrodes (outside diameter, 76 µm) around the anterior and posterior trunks, about 2–3 cm above the gastroesophageal junction and above the accessory and celiac
branches of the vagus nerve. These stimulating electrodes were loosely sutured to the esophagus to limit displacement. At the end of each experiment, an overdose of anesthetic was administered to kill the animals. All experimental procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Recording of single nodose neuronal activity**

Rats were placed in a small Kopf animal stereotaxic frame. Rectal temperature was maintained with a special heating pad. The right nodose ganglion was exposed by a short dorsal approach. Using an operating microscope, the ganglion sheath was removed and separated from the adjacent cervical sympathetic trunk and carotid artery. Protease (type XIV, 0.3 mg/mL) was applied to the ganglion for 15 min to facilitate the removal of the ganglion sheath. In this process little or no protease came into contact with the nodose neuron and should not affect the expression or function of the CCK or leptin receptors. The recording microelectrodes were pulled from glass capillaries (A-M Systems, Everett, WA) using a micropipette puller and microelectrode beveler to obtain tips which yielded a resistance of 50–70 MΩ. The beveled glass micropipette filled with 1.0 M KCl was lowered into the nodose ganglion. Once a nodose ganglion neuron activated by the electrical vagal stimulation was identified, the responses of that neuron to intraarterial injections of CCK-8 and leptin were examined. A reference electrode was placed on a skin incision near the recording electrode. Only gastrointestinal C-fibers were recorded; these were identified according to the following parameters measured in response to electrical stimulation of the abdominal vagus nerve: latency, 60–80 ms; conduction distance between the stimulating electrode and the nodose ganglion, 0.06 m; and conduction velocity, <1.0 m/s. Extracellular recordings of the neuronal discharges were amplified by an A-M System
high-input impedance preamplifier, monitored with an oscilloscope and audio monitor, and displayed and stored on an IBM-compatible computer using Axon software. Following 30 min of stabilization period, the basal discharge was monitored for 2 min to confirm the stability of the basal firing frequency. Consistent monitoring of each neuron was ensured by careful study of the firing pattern produced by each neuron and also by studying the amplitude and waveform of each spike. This recording technique has been well established in our previous studies (14,16,18,42).

**Vagal nodose neuronal responses to CCK-8 and leptin**

The superior pancreaticoduodenal artery, which mainly supplies the duodenum, was cannulated. The common hepatic artery was exposed and temporarily ligated. The gastroduodenal artery was punctured at its junction with the common hepatic artery. The catheter was inserted and threaded into the superior pancreaticoduodenal artery about 0.5 cm past the gastroepiploic artery. The catheter was fixed in place with a suture, and the puncture hole was sealed with cyanoacrylate glue. Once a stable single nodose neuronal recording was established, 1-min basal spontaneous firings were recorded. The neuronal responses to bolus injections (100 µl) of various doses of CCK-8 (10, 60, and 120 pmol) and leptin (225, 500 pmol) given over 2 seconds into the superior pancreaticoduodenal artery were examined. The different doses of CCK-8 and leptin were delivered randomly in each study. Recording was continued for 3 min, with 15 min between each dose. Nodose ganglia neurons that exhibited a positive response to stimulation by CCK-8/leptin were tested for their responses to intraluminal perfusion of 5-HT and gastric distention as described below.

**Mucosal vagal afferent fibers vs. muscle tension receptors**
Nodose ganglion neurons that respond to intraarterial infusion of CCK-8 and leptin may innervate the muscle layer and/or the mucosa. It is well recognized that nodose ganglion neurons that respond to distention innervate the muscle layer, whereas those that respond to intestinal chemostimulation (e.g., 5-HT) innervate the mucosa (16). In the present study, to characterize the nodose neurons activated by CCK-8/leptin, we examined the responses of CCK-8/leptin–sensitive neurons to gastric distention and luminal perfusion of 5-HT using previously described methods (16). Balloons for gastric distention were placed surgically. A 1.5- to 2.0-cm–long left lateral epigastric incision was made to expose the stomach. Latex balloons (2.0–2.5 cm diameter) were inserted through the fundus into stomach. The polyethylene tubing for balloon inflation was exteriorated at the back of the neck. The stomach was filled with prewarmed saline to generate a baseline pressure of 3 mmHg. To identify nodose neurons that were sensitive to mechanical distension, a distention control device was used to evoke a distention pressure of 60 mmHg. Intragastric pressure was monitored by connecting the catheter to a pressure transducer by way of a three-way stopcock. To identify vagal afferent fibers innervating the mucosa, we examined the vagal nodose neuronal response to intestinal perfusion of 5-HT. A 20–30 cm segment of the small intestine, including the entire duodenum and proximal jejunum, was isolated between two cannulas positioned at 4 cm (PE 60; 0.76 mm inner diameter and 1.22 mm outer diameter) and 24 cm (PE 190; 1.19 mm inner diameter and 1.7 mm outer diameter) from the pylorus. After a 15-min basal period, 1.5 mL 5-HT (10^{-5} M) was perfused into the duodenum over 1 min. Free intestinal drainage prevented an increase in intraluminal pressure. Control and test solutions were delivered separately over 1 min and washed out with isotonic saline. Recording of the nodose ganglia neurons was continued for 5 min with a 30-min resting period between experiments.
Interaction between CCK and leptin

Because a group of neurons possessing CCK-A receptors also responded to intraarterial infusion of leptin, we examined the nature of the interaction between CCK-8 and leptin in vagal primary afferent neurons. Electrophysiological recordings of nodose neurons in response to intraarterial injection of CCK-8, leptin, and CCK-8 combined with leptin were performed. After a 1-min basal recording, the neuronal responses to bolus injections of CCK-8 (10, 60, and 120 pmol) were examined. When a CCK-responsive nodose ganglia neuron was identified, it was tested for its responsiveness to leptin (225 pmol) as described in the previous section. Neurons that exhibited an increased basal discharge frequency (>4 impulses per 20 s) were not tested further. After a 5-min resting period, an intraarterial injection of CCK-8 (120 pmol) combined with leptin (225 pmol) was administered. Recording was continued for 3 min, with 5 min between each dose. Neurons that did not respond to both CCK-8 and leptin were discarded. A different neuron was identified after electrical stimulation of the subdiaphragmatic vagus nerve, and the experimental procedure was repeated.

JMV-180 studies

JMV-180 has been shown to interact with both classes of pancreatic CCK-A receptors – acting as an agonist in high-affinity states and an antagonist in low-affinity states. To identify the vagal CCK-A receptor affinity state that mediates the synergistic effect of CCK and leptin in the vagal afferent neurons, we performed JMV-180 studies. JMV-180 (2 and 5 µg) was injected intraarterially 30 sec after the administration of CCK-8 and leptin.

Data analysis
Single neuronal responses were examined using the Datapac 2000 software system (Run Technologies, Laguna Hills, CA). The prestimulus discharge frequency was assessed for 1 min to quantify the resting discharge. The discharge frequency after the administration of CCK-8 or leptin, after the intraluminal perfusion of 5-HT, and after gastric distention was measured for 3 min. Time histograms were constructed for the 1-min period before stimulation and for the 2-min period after stimulation. The mean and standard deviation (SD) of nodose neuronal firing during the 30-sec control period was compared with the maximal activity after administration of stimulus. A positive response was defined as an increase of $\geq 2$ SD in the maximal activity after the infusion of CCK-8 or leptin or a combination of the two peptides compared to the mean firing rate in the control period. Results were expressed as means ± SEM. Statistical significance was determined using the appropriate Student $t$ test (paired or unpaired) and the Newman-Keuls test when multiple comparisons were made (InStat Biostatistics 2.01. GraphPad Software, La Jolla, CA). $P < 0.01$ was considered statistically significant.

**Immunocytochemistry**

*Tissue preparation.* Immunocytochemistry studies were performed as described previously (14, 33). A transcardial perfusion was administered 1 h after termination of the intraperitoneal administration of CCK-8, leptin, JMV-180, or leptin with CCK-8 plus JMV-180. Tissue was rinsed first with ice-cold heparinized PBS (10 mmol/L, pH 7.2) and subsequently with 0.2 L of fixative containing 4% paraformaldehyde, 0.2% picric acid, and 0.35% glutaraldehyde in phosphate buffer (0.1 mol/L, pH 7.4). The left and right nodose ganglia were removed and placed in the same fixative for 2 h at 22°C and then in 25% sucrose in PBS (0.1 mol/L) overnight at 4°C. The ganglia were cut in longitudinal sections (8–10 μm) using a Thermo Scientific
cryostat (HM 550). The sections were mounted on gelatin-coated slides. In general 50 sections were collected. The sections were separated into five series and stored at \(-70^\circ\text{C}\). The first set (10 \(\mu\text{M}\)), which comprised every fifth section, about 10 sections total, was used for c-Fos immunohistochemical staining. The remaining sets from each ganglion were used for CCK-A receptor and leptin receptor immunocytochemistry and c-Fos staining. The CCK-A receptor antibody (SC-16172) used for our immunohistochemical staining is an affinity purified goat polyclonal antibody raised against the N-terminus 20 amino acids peptide of human CCK-A receptor. The leptin receptor antibody (B3) (SC-8391) is a mouse monoclonal antibody specific for the epitope between amino acid 870-894 of the short form OB-Rb of mouse. This antibody detects both the short and long form of leptin receptor. Both antibodies purchased from Santa Cruz Biotechnology, Inc. are recommended for the detection of their respective receptors of mouse, rat and human origins. To estimate the total number of neurons in each ganglion, one set from each ganglion was counterstained with cresyl violet.

**c-Fos immunocytochemistry.** Immunohistochemical staining for c-Fos expression was performed. The sections were washed three times in wash buffer (PBS containing 0.3% Triton X-100) and immersed in a blocking solution (5% normal goat serum, 1% BSA, and 0.5% Triton X-100 in PBS) for 20–30 min to inhibit nonspecific binding. The sections were then incubated in a humid chamber for 48 h at 4°C with c-Fos rabbit polyclonal antibody (Ab-5) (PC38, Calbiochem) diluted to 1:2,000. After incubation, the sections were washed in PBS buffer and incubated for 1 h at room temperature with species-specific fluorophore-conjugated secondary antibodies (goat anti-rabbit conjugated to Cy3 [Jackson ImmunoResearch Laboratories, West Grove, PA]). For controls, the primary antiserum was omitted and the sections were incubated in
normal goat serum. Antibody specificity was verified by incubation with antisera preabsorbed with synthetic c-Fos protein (10 μmol, c-Fos peptide-2, lot no. 954701; Oncogene Science) for 1 h at room temperature. This procedure blocked all immunocytochemical staining.

**Immunocytochemical staining for c-Fos with CCK-A and leptin receptors.** Double or triple immunocytochemical staining for c-Fos plus CCK-A receptors and/or leptin receptors (Ob-R) was performed. Sections were washed in PBS buffer three times and incubated with c-Fos rabbit antibody (Ab-5) obtained from Calbiochem diluted in 1:2,000 and one or two of the following antibodies: goat anti-CCKAR polyclonal antibody (1:200, SC-16172, Santa Crutz, CA) and/or mouse anti-OB-Rb (B-3) monoclonal antibody (1:250, SC-8391, Santa Crutz). The incubation was performed in a humid chamber for 16–48 hours at 4°C. After incubation, sections were washed three times in PBS buffer and incubated for 1 h at room temperature in species-specific fluorophore-conjugated secondary antibodies (Cy3, AMCA [Jackson ImmunoResearch Laboratories] and Alexa Fluor 488 [Molecular Probes, Invitrogen, Carlsbad, CA]). Sections were rinsed thoroughly in wash buffer, mounted on gelatin-coated slides, and coverslipped. Sections were examined with a Zeiss KS400 LSM confocal laser scanning microscope. Specificity of immunohistochemical labeling was verified with either the omission of the primary antibody or inclusion of blocking peptides for CCK-AR or OB-Rb staining (Santa Cruz Biotechnology, Inc.)

**Quantification and statistical analysis.** c-Fos-, CCKAR- and OB-Rb–immunoreactive (IR) cells were counted in sections cut longitudinally through the entire length of the nodose ganglion according to previously published methods from our laboratory (39). Representative cumulative numbers of immunoreactive cells were obtained by counting one set of each section, i.e. every
fifth section (10 µM thick) from both the left and right nodose ganglia, under ×40 magnification. In our studies, we counted every fifth section which are 50 µM apart. This is greater than the diameter (20-30 µM) of most neurons in the rat nodose ganglion. This would greatly diminish double counting errors. To be counted as either a c-Fos–positive or an unlabeled neuron, the nucleus had to be clearly visible; therefore, fractions of labeled neurons that did not contain a nucleus were not counted. Each section was counted a minimum of two times: the first by the primary investigator; the second, a blind count by an independent observer. Data were presented as means ± SE and evaluated with a one-way analysis of variance (ANOVA). Of the data from 56 rats, data from 2 rats were not used in the final analysis because of problems encountered with sectioning on immunocytochemical processing.
Results

Effects of CCK-8, leptin, and gastric distention on nodose neuronal activity

Data were collected from 264 recordings of single nodose ganglia neurons in 54 rats. All 264 units activated by electrical stimulation of the subdiaphragmatic vagus nerve were tested with intra superior pancreaticoduodenal artery injection of CCK-8 (10, 60, and 120 pmol) and leptin (225 and 500 µg). Of 264 neurons, 82 responded to intraarterial injections of CCK-8 and/or leptin. All units were either silent or displayed very low spontaneous activity (0–1.5 impulses per 20 s) before the CCK-8 and leptin infusions. In all cases, the threshold dose of CCK-8 was ≥ 5 pmol. The short latency of the CCK-8 response (i.e., 1.0 ± 0.3 sec) was likely the result of the intraarterial administration, as CCK-8 reached the intestinal mucosa soon after injection. Three groups of vagal afferent neurons were identified among the 82 responsive neurons. Group 1 (24 neurons) responded to CCK-8, but not leptin. Of these 24 neurons, 8 responded to CCK-8 at doses of 10 and 60 pmol; the neuronal firing rate increased from 0 ± 0.5 to 5.5 ± 1 and 19 ± 2.5 impulses per 20 s, respectively. These neurons did not respond to a high dose of CCK-8 (120 pmol), leptin, or gastric distention (Fig. 1), but they did respond to luminal 5-HT. This indicates the presence of high affinity CCK-ARs. Typically this group of receptors are activated by low concentrations of CCK but inhibited by high concentrations (18). The other 16 neurons, although unresponsive to the low doses of CCK-8, were activated by a higher dose of CCK-8 (120 pmol); the neuronal firing rate increased from a basal of 0 ± 1 to 22 ± 2 impulses per 20 sec. Of these 16 neurons, 12 were also activated by gastric distention. None of these 16 neurons were activated by JMV-180 or luminal 5-HT. Group 2 (22 neurons) responded to leptin at doses of 225 and 500 pmol; the firing rates increased from a basal of 0 ± 0.5 to 11.5 ± 1.0 and 35 ± 5 impulses per 20 sec, respectively (Fig. 2). These neurons were not activated by CCK-8 (up to 120 pmol) (Fig. 2).
Of these 22 neurons, 10 responded to gastric distention (Fig. 2) and the remaining 12 responded to luminal 5-HT. Group 3 neurons (36 neurons) responded to both CCK-8 (120 pmol) and leptin (Fig. 3). Of these 36 neurons, 30 neurons were activated by gastric distention (Fig. 3) but not luminal 5-HT.

**Interaction between CCK-8 and leptin**

In separate studies, data were collected from 108 recordings of single nodose ganglia neurons activated by electrical stimulation of the subdiaphragmatic vagus nerve in 20 rats. Infusion of a high dose of CCK-8 (120 pmol) activated 18 neurons; neuronal firing increased from a basal of 0 ± 0.6 to 19 ± 3 impulses per 20 s. These neurons were then tested for their responsiveness to leptin. Of the 18 neurons that responded to the high dose of CCK-8, 12 were activated after the infusion of leptin (Fig. 4). Leptin at doses of 225 and 500 pmol increased neuronal discharges from a basal of 1 ± 0.4 to 13.1 ± 1.6 and 23.0 ± 1.0 impulses per 20 s, respectively. Then we examined the interaction between CCK-8 and leptin. A combination of CCK-8 (120 pmol) and leptin (225 pmol) increased the neuronal firing rate from a basal of 1 ± 0.5 to 59 ± 3.8 impulses per 20 s (Fig. 4, Fig. 6). The total increase in neuronal discharge was greater than the sum of neuronal discharges stimulated by CCK-8 (23 ± 2 impulses per 20 s) and leptin (18 ± 0.5 impulses per 20 s) alone (Fig. 4, Fig. 6), suggesting a potentiation effect between these two peptides on vagal nodose neuronal activities. All these neurons responded to gastric distention (Fig. 4) but not luminal 5-HT. We also performed experiments and demonstrated that subthreshold dose of CCK-8 (60 pmol) for low affinity CCKAR and leptin (112 pmol) when administered together caused an increase of nodose neuron firing from a basal of 1±0.2 to 35±5
impulses per 20 s (n=6). This suggests that a potentiation effect between CCK-8 and leptin may also occur at subthreshold doses.

**Effects of CCK-JMV-180 on nodose neuronal activity stimulated by CCK-8 combined with leptin**

To identify the vagal CCK-A receptor affinity state that mediates the synergistic interaction between CCK-8 and leptin, we performed additional studies in 42 rats. Of 216 neurons activated by electrical stimulation of the subdiaphragmatic vagus nerve, 46 neurons were stimulated with an intraarterial injection of CCK-8 (120 pmol). Nodose neuronal firing increased from a basal of 0 ± 0.7 impulses to 18 ± 0.7 impulses per 20 s. Of the 46 neurons, 22 were activated after administration of leptin (225 pmol); firing increased from a basal 1 ± 0.5 to 13 ± 2.0 impulses per 20 s. All 22 neurons failed to respond to JMV-180 (2.0 and 5.0 µg). The effect of JMV-180 on the potentiation between CCK-8 and leptin on nodose ganglia neurons was investigated. Tests were performed on 14 neurons. The combined administration of CCK-8 (120 pmol) and leptin (225 pmol) increased neuronal firing from a basal of 1 ± 0.5 impulses to 51 ± 3 impulses per 20 s. Infusion of JMV-180 (5.0 µg) 30 s after the administration of CCK-8 plus leptin blocked the synergistic interaction observed after the administration of CCK-8 and leptin together (Fig. 5E, 6). The mean discharge frequency of nodose ganglia neurons in response to CCK-8, leptin, and CCK-8 plus leptin is presented in Figure 6. To rule out the possibility that the inhibitory action of JMV-180 is due to desensitization we showed that administration of CCK-8 (120 pmol) instead of JMV-180 (5.0 µg) did not cause a reduction of neuronal firing evoked by a combination of CCK-8 plus leptin (Fig 5F).
Expression of CCKAR and OB-Rb in rat nodose ganglion

Double immunofluorescence labeling was performed to examine the expression of CCKAR and OB-Rb in the rat nodose ganglion (n = 3). Representative images are shown in Fig. 7A–C. About 74% neurons were immunoreactive to either CCKAR or OB-Rb. Among the total ganglion neurons, 37.2 ± 3.5% expressed CCKAR, 18.0 ± 5.6% expressed OB-Rb, and 18.7 ± 2.8% expressed both CCKAR and OB-Rb (Fig. 7A–D).

Effects of JMV-180 on CCK- and leptin-induced c-Fos expression in rat nodose ganglion

First, we examined the c-Fos expression in the rat nodose ganglion treated with CCK-8 and leptin. A group of rats (n = 3) were given an intraperitoneal (IP) injection of CCK-8 and leptin at doses of 3.5 µg/kg and 120 µg/kg, respectively. These doses of CCK-8 and leptin were used previously by other investigators to demonstrate synergistic interaction between leptin and CCK to reduce short-term food intake in lean mice (1). The rats were sacrificed 60 min after the injection. c-Fos–IR neurons in the nodose ganglion that exhibited immunoreactivity for either CCKAR or OB-Rb were determined by triple immunostaining (Fig. 8A). The CCK-8 and leptin injection produced c-Fos expression in 13.7 ± 1.1% of the total ganglion neurons. Among these c-Fos–positive neurons, 3.2 ± 0.3% expressed CCKAR, 1.5 ± 0.3% expressed OB-Rb, and 6.1 ± 0.6% expressed both CCKAR and OB-Rb (Fig. 8A–C).

We next examined the effects of JMV-180 on c-Fos expression evoked by CCK-8 and leptin in the nodose ganglia. In this group of rats (n = 3), JMV-180 (350 µg/kg, IP) was given 15 min before a combined injection of CCK-8 (3.5 µg/kg, IP) and leptin (120 µg/kg, IP). The animals were sacrificed for immunostaining 60 min after the injection of CCK-8 and leptin. Administration of JMV-180 did not affect the immunostaining of CCKAR and OB-Rb (data not
shown). Microphotograph images of triple fluorescence labeling are shown in Fig. 8A and B. However, the percentage of total vagal nodose ganglia neurons that exhibited c-Fos–IR in response to CCK-8/leptin administration was significantly decreased in rats treated with JMV-180 (Fig. 8).

CCK-8 (3.5 µg/kg) and leptin (120 µg/kg) induced c-Fos expression in 13.7 ± 1.1% of the total ganglia neurons. JMV-180 (350 µg/kg, IP) reduced c-Fos expression to 4.9 ± 1.0% (Fig. 8C). Specifically, JMV-180 significantly suppressed c-Fos expression in neurons expressing CCKAR (1.0 ± 0.2% vs. 3.2 ± 0.3%; P = 0.0073) and those expressing both CCK-AR and OB-Rb (1.2 ± 0.2% vs. 6.1 ± 0.6%; P = 0.0085), but not in neurons expressing OB-Rb (1.5 ± 0.3% vs 1.1 ± 0.3%; P = 0.290). Similarly, JMV-180 had no effect on c-Fos expression in neurons expressing neither CCKAR nor OB-Rb (2.8 ± 0.8% vs 1.7 ± 0.4%; P = 0.30).
Discussion

Both CCK and leptin are important satiety factors mediating eating behavior and regulating body weight. In the rodents, numerous studies demonstrated that leptin is a key signaling molecule responsible for long-term satiety and energy balance. Mutations that cause defective leptin secretion or abnormal leptin receptor signaling result in obesity in Ob/Ob mice (2) and in humans (24). There are also data from humans showing a role for leptin in satiety and satiation. In patients with lipodystrophy and leptin insufficiency (22), after 4 months of leptin administration, there was normalization of serum leptin accompanied by a 53% reduction of the time for induction of satiety and 219% increase in the length of time subjects remained satied.

Recent studies provided evidence for a synergistic interaction between leptin and CCK leading to short-term (first 3 hours after eating) reduction of food intake (1). In fact, the satiety action of CCK was entirely dependent on leptin signaling. In a preliminary study, we showed that in the Ob/Ob mice which are leptin deficient, CCK injection alone did not affect short-term food intake, whereas the inhibitory action of leptin in hours 5-7 was similar to lean mice (26). Co-administration of CCK with leptin restored the satiety action of CCK. It reduced short-term food intake similar to that observed in the lean mice. Currently, little is known about the site(s) and mechanism(s) of interaction between leptin and CCK on satiety control.

Although there is evidence that both CCK and leptin may act centrally to regulate food intake, the primary site(s) of satiety action of these two peptides is likely on the peripheral terminals of vagal afferent fibers supplying the gastrointestinal tract (1,8,21,33,36). CCKAR have been well demonstrated in the rat vagus nerve (40). Nerve ligation studies show that these receptors are transported peripherally from the nodose ganglia (40). Furthermore, CCK binding and axonal transport are evident in all abdominal vagal branches (23,25,35). Similarly, the long
form of leptin receptors (OB-Rb) has been identified in the vagal nerve (6,7). Burdyga et al. reported that many of the vagal afferent neurons that express CCKAR may also express OB-Rb (6). Using isolated nodose ganglia neurons, it was demonstrated that CCK activated both A- and C-type vagal afferent neurons (34). Further, leptin rapidly increased cytosolic calcium in vagal afferent neurons and this signal was synergistically enhanced by the presence of CCK (29). Using patch-clamp electrophysiological studies, these investigators demonstrated that leptin reversibly depolarized a subpopulation of cultured nodose neurons; many of which were also activated by CCK (31). These function studies suggest that vagal afferent neurons are potential targets for leptin–CCK interaction for the control of satiety. It should be noted that all these observations were made on cell culture systems, which may induce artifacts in receptor expression or signal transduction molecules, leading to erroneous interpretations. The extrapolation of in vitro observations to the intact in vivo system is often problematic because of specific physical and chemical conditions used in the cell culture system. Furthermore, the separation of neurons from their extrinsic inputs may have a significant impact on the properties of the neuronal membranes.

Electrophysiology and immunocytochemistry studies have revealed clearly that the nodose ganglia are not homogenous; they contain subpopulation of neurons that express different groups of receptors (5,28). Furthermore, similar to CCKAR in the rat pancreatic acini, vagal CCKAR occur in both low- and high-affinity states (19,27). The objectives of our study were to determine the distribution pattern of high- and low-affinity CCKAR and their relationship to leptin receptors on the vagal nodose neurons.

We used doses of CCK and leptin which produced blood levels within physiological ranges (13,30). Our in vivo electrophysiological studies demonstrated that 31% (82 of 264
neurons) of the vagal afferent neurons, which innervate the gastrointestinal tract, were sensitive to either CCK-8 and/or leptin. These neurons have differential sensitivity to different doses of CCK-8 and leptin. Of this group of neurons, 29% (24 of 82 neurons) responded to CCK-8 but not leptin. One-third (8 of 24 neurons) of these were activated by low-dose CCK-8 ($\leq 60$ pmol), and the remaining two-thirds were activated only by high-dose CCK-8 ($\geq 120$ pmol). The neurons activated by low-dose CCK-8 were also uniformly responsive to the high-affinity CCK receptor agonist, JMV-180. In contrast, the neurons that reacted to high-dose CCK-8 were insensitive to JMV-180. This suggests that high- and low-affinity CCK receptor states occur in different populations of nodose ganglia neurons.

The gastrointestinal sensory receptors usually are classified into three groups: mucosal, muscle, and serosal receptors. Mucosal receptors lie in or immediately below the mucosal epithelium and detect the physical and chemical nature of luminal contents. Muscle receptors are located deep in the musclaris externae and are influenced by changes in muscle tension. In the current study, intraintestinal perfusion of 5-HT was used to identify vagal mucosa afferent neurons (12,15,39,41). All the neurons that responded to low-dose but not high-dose CCK-8 were sensitive to luminal 5-HT perfusion. This suggests the presence of high-affinity CCKAR on vagal afferent fibers innervating the intestinal mucosa but not on those terminating on muscle fibers. It is likely that this group of neurons is involved in CCK-stimulated pancreatic secretion, which has been shown to be mediated by high-affinity vagal CCKAR (11). On the other hand, vagal neurons that responded only to high-dose CCK were sensitive to gastric distention, suggesting that this group of nodose vagal afferent neurons that contain low-affinity receptors are also muscle tension receptors. Our observations support the findings of Schwartz et al. (32)
who reported that JMV-180 completely blocked the gastric mechanosensitive vagal response to arterial infusion of CCK-8.

Our in vivo electrophysiological studies showed that 27% (22 of 82 neurons) of the vagal afferent neurons innervating the gastrointestinal tract were activated by leptin but not CCK-8. About 50% of these neurons also responded to gastric distention. It is interesting to note that the administration of leptin alone does not seem to affect short-term feeding; however, 5–6 hours after its administration, food intake decreases (1). In addition, to serve as a long-term regulator of nutrient intake, leptin may regulate adiposity and body weight. Some of these functions may be regulated by leptin’s actions in the hypothalamus and/or the vagal afferent neurons. It is conceivable that this group of neurons bearing leptin receptors but not CCKAR may be involved in the regulation of some of these functions.

Of the vagal afferent neurons innervating the gastrointestinal tract, 44% (36 of 82 neurons) were activated by both leptin and CCK-8, and most neurons in this group also responded to gastric distention. These neurons responded to high- but not low-dose CCK-8. The synergistic interaction with leptin was blocked by JMV-180, suggesting that these CCKAR are in the low-affinity state. These data provide a neurochemical basis to explain the observation that JMV-180 dose-dependently reverses the effect of CCK-8 on satiety (38).

The electrophysiological observations were corroborated by the immunohistochemical studies. We performed c-Fos expression studies in conjunction with double labeling of CCKAR and OB-Rb to characterize the neurochemical phenotype of c-Fos–positive nodose ganglia neurons. Expression of the immediate-early gene product c-Fos has been widely used as a marker of neuroactivation in the brain. Because Fos expression is relatively rapid and easily detected by immunohistochemical staining of the cell nucleus, this method has been used
extensively in in vivo studies to map postsynaptic activation of the nervous system (4,9,10). Fos expression in the vagal primary afferent neurons has been reported in rats after administration of secretin (16) and in response to luminal nutrient stimulation (39). In the present study, we showed that intraperitoneal administration of CCK-8 and leptin increased c-Fos protein expression in the nodose ganglia neurons of rats. This increase was reduced by the administration of JMV-180, which acts as an antagonist for the low-affinity CCK receptor. This action was receptor specific, as JMV-180 only reduced c-Fos expression in neurons that expressed CCKAR, or those that expressed both CCKR and leptin receptors, but not those expressing only leptin receptors. c-Fos expression in neurons expressing CCKAR was reduced by two-thirds in the presence of JMV-180, suggesting that this subgroup of neurons expresses low-affinity CCK-A receptors. The remaining one-third, which were resistant to the inhibitory action of JMV-180, likely express high-affinity CCKAR. The most dramatic suppression by JMV-180 of c-Fos expression was observed in neurons that expressed both CCK-A and leptin receptors. This confirmed that CCKAR that coexpress with leptin receptors are low-affinity CCK receptors.

In summary, using electrophysiological and immunohistochemical studies, we showed that low- and high-affinity CCK-A receptors are expressed in different populations of nodose ganglia. The high-affinity CCK receptors are localized in mucosal vagal afferent fibers, whereas the low-affinity CCK receptors are found in fibers innervating the muscle layers. These fibers are also mechanosensitive. A subgroup of nodose neurons that expressed low-affinity CCK receptors also expressed leptin receptors. This particular group of neurons also responded to gastric distention. Interaction between low-affinity CCK-A receptors and leptin receptors in these neurons likely facilitates leptin mediation of short-term satiety. The present data provide a
neurochemical basis for the synergistic interaction between CCK and leptin to regulate feeding behavior.
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Disclosures

The authors declare no conflict of interest.
References


Figure Legends

Figure 1. Response of a nodose ganglia neuron to intra superior pancreaticoduodenal artery infusions of CCK-8 and leptin and gastric volume distention. Intraarterial infusion of CCK-8 at a dose of 60 pmol (A) but not 120 pmol (B) produced an increase in nodose neuronal discharge frequency, which indicates the presence of high-affinity CCK-A receptors on a subgroup of vagal primary afferent neurons. (C) Administration of leptin at 225 pmol did not change the neuronal activity of this neuron. (D) The same neuron also failed to respond to gastric distention.

Figure 2. Response of a nodose ganglia neuron to intra superior pancreaticoduodenal artery infusions of CCK-8 and leptin and gastric volume distention. Intraarterial infusion of CCK-8 at doses of 60 pmol (A) and 120 pmol (B) did not stimulate afferent firing of this neuron. (C) Administration of leptin at 225 pmol produced a marked increase in vagal afferent discharge in the same neuron. (D) The same neuron responded to gastric distention, which suggests leptin receptors reside on vagal afferents, and they may in fact innervate the gastric muscle layer.

Figure 3. Response of another nodose ganglia neuron to intra superior pancreaticoduodenal artery infusions of CCK-8 and leptin and gastric volume distention. Intraarterial infusion of CCK-8 at a dose of 10 pmol (A) did not stimulate afferent firing of this neuron. Administration of CCK-8 at a dose of 120 pmol (B) and leptin at 225 pmol (C) stimulated afferent firing. (D) Gastric distention produced a marked increase in vagal afferent discharge in the same neuron. Waveforms of the recorded unit after leptin treatment and gastric distention are shown in (E) and (F) respectively. The two waveform are superimposed (E and F).
indicating recording were made from the same unit.

**Figure 4. Response of a nodose ganglia neuron to intra superior pancreaticoduodenal artery infusions of CCK-8 combined with leptin.** Intraarterial infusion of CCK-8 (10 pmol) (A) did not stimulate vagal nodose neuronal firing. Administration of CCK-8 at 120 pmol (B) and leptin (225 pmol) (C) increased the neuronal discharge frequency. (D) Administration of leptin in combination with CCK-8 resulted in a marked increase in neuronal firing, suggesting a potentiation effect between these two peptides. (E) This neuron also responded to gastric distention.

**Figure 5. Interaction between CCK-8 and leptin on nodose neuronal firing, and the effect of JMV-180 on this interaction.** Intraarterial infusion of CCK-8 (10 pmol) (A) did not stimulate vagal nodose neuronal firing. CCK-8 at 120 pmol (B) and leptin at 225 pmol (C) increased the neuronal discharge frequency. (D) A synergistic effect was observed when CCK and leptin were infused together. (E,F) Administration of JMV-180 but not CCK-8 prevented this potentiation effect, which suggests that low-affinity CCK-A receptors are coexpressed with leptin receptors in rat nodose ganglia.

**Figure 6. Discharge of nodose ganglia neurons in response to intraarterial injection of CCK-8 and leptin, and the effect of JMV-180 on the interaction between CCK-8 and leptin.** Data were collected from 264 recordings of neurons activated by electrical stimulation of the subdiaphragmatic vagus nerve. Of these, 24 neurons responded to CCK, another 22 neurons responded to leptin and 36 neurons responded to both CCK-8 and leptin. Intraarterial injection of
CCK (120 pmol) enhanced the neuronal responses to leptin (225 pmol). Furthermore, administration of the low-affinity CCK-A receptor antagonist JMV-180 abolished the enhanced responses induced by CCK-8 combined with leptin. *P < 0.01 (compared with the sum of CCK + leptin alone). **P < 0.05 (compared with CCK-8 + leptin).

**Figure 7. CCKAR and Ob-R expression in rat nodose ganglion.** Double fluorescence labeling of rat nodose ganglia shows expression of CCKAR and OB-Rb. (A) CCKAR are stained with green fluorescent Alexa Flour 488. (B) OB-Rb are stained with red fluorescent Cy3. (C) Nodose ganglia sections of A and B merged; the neurons expressing CCKAR and OB-Rb are double labeled and show as yellow. Scale bar: 50 μm. (D) Histogram shows the percentage of the total number of nodose ganglia neurons that exhibited immunoreactivities for CCKAR, OB-Rb, or both CCK-AR and OB-Rb (n = 3).

**Figure 8. Effects of JMV-180 on CCK- and leptin-induced c-Fos expression in rat nodose ganglion.** Triple fluorescence labeling of rat nodose ganglia shows c-Fos–positive neuronal nuclei (red), CCKAR (blue), and OB-Rb (green). (A) c-Fos expression in nodose ganglion in response to intraperitoneal (IP) injection of CCK-8 (3.5 μg/kg) and leptin (120 μg/kg). (B) c-Fos expression in nodose ganglion treated with JMV-180 (350 μg/kg, IP) 15 minutes before IP injection of CCK-8 (3.5 μg/kg) and leptin (120 μg/kg). JMV-180 reduced c-Fos–IR in the nodose ganglion. Scale bar: 100 μm. (C) Histogram shows percentage of neurons that exhibited c-Fos immunoreactivities over the total number of neurons in the nodose ganglion (n = 3). Note that JMV-180 significantly reduced c-Fos expression in the neurons expressing CCKAR and
those expressing both CCKAR and OB-Rb. \( *P < 0.01 \) compared to no pretreatment with JMV-180.
Fig. 1

A

CCK-8 60 pmol

B

CCK-8 120 pmol

C

Leptin 225 pmol

D

Gastric Distention 10 sec

Fig. 1
Fig. 2

A

CCK-8 10 pmol

B

CCK-8 120 pmol

C

Leptin 225 pmol

D

Gastric Distention

10 sec
Fig. 3

A

CCK-8 10 pmol

B

CCK-8 120 pmol

C

Leptin 225 pmol

D

Leptin treatment

Gastric Distention

E

Superimposed waveforms from E and F

Leptin treatment

F

Gastric distension

Superimposed waveforms from F and E

10 sec
Fig. 4

A

CCK-8 10 pmol

B

CCK-8 120 pmol

C

Leptin 225 pmol

D

CCK-8 120 pmol + Leptin 225 pmol

E

Gastric Distention

10 sec
CCK-8 10 pmol

CCK-8 120 pmol

Leptin 225 pmol

CCK-8 120 pmol + Leptin 225 pmol

CCK-8 120 pmol + Leptin 225 pmol + JMV-180 5.0μg

CCK-8 120 pmol + Leptin 225 pmol + CCK-8

Fig. 5
Fig. 7
Fig. 8

CCK-8+Leptin
JMV180 plus CCK8+Leptin

% of expression

Total c-Fos  c-Fos/CCK-AR  c-Fos/Ob-R  c-Fos/CCK-AR+Ob-R  C-Fos only