Functional Compensation between Cholecystokinin-1 and -2 Receptors in Murine Paraventricular Nucleus Neurons*

Shahid Mohammad‡1, Tomoya Ozaki‡1, Kouhei Takeuchi‡5, Katsuya Unno§, Kurumi Yamoto§, Eri Morioka‡, Soichi Takiguchi‡, and Masayuki Ikeda‡§2

From the 1Graduate School of Innovative Life Science and the 2Graduate School of Science and Engineering, University of Toyama, 3190 Gofuku, Toyama-city, Toyama 930-8555 and the 3Institute for Clinical Research, National Kyushu Cancer Center, 3-1-1 Notame, Minami-ku, Fukuoka 811-1395, Japan

Background: Cholecystokinin (CCK)-1 receptor ligands control satiety. Normal intake behaviors in the receptor gene knock-out mice, however, raise questions for CCK-mediated satiety control.

Results: CCK-1 receptor gene knock-out facilitated CCK-2 receptor signaling in the posterior paraventricular nucleus and switched receptor subtypes responsible for satiety control.

Conclusion: Synergic interaction between CCK-1 and -2 receptors guarantees regular intakes.

Significance: Compensatory receptor functions shed light on CCK-mediated satiety controlling mechanism.

Cholecystokinin (CCK) and its receptor subtypes CCK-1 and -2 have diverse homeostatic functions. CCK-1 and -2 receptors share a common phosphatidylinositol signaling pathway, yet little is known regarding their possible functional coupling. We focused on CCK-mediated Ca2+ signaling in parvocellular paraventricular nucleus (PVN) cells, which control satiety and other autonomic functions. Analysis of mouse hypothalamic slices demonstrated that the general CCK receptor agonist CCK-8s (10 nM) triggered Ca2+ transients most significantly in the posterior subregion of the PVN (PaPo). This 10 nM CCK-8s-induced response was absent in CCK-1 receptor knock-out (CCK1R−/−) slices, showing that the response is mediated by CCK-1 receptors. CCK-8s concentrations higher than 30 nM triggered a Ca2+ rise similarly in wild-type and CCK1R−/− slices. The large CCK-8s (100 nM)-induced Ca2+ responses in CCK1R−/− slices were blocked by a CCK-2 receptor antagonist (CI-988), whereas those in wild-type slices required a mixture of CI-988 and longlumide (a CCK-1 receptor antagonist) for complete antagonism. Therefore, CCK-1 and -2 receptors may function synergistically in single PaPo neurons and deletion of CCK-1 receptors may facilitate CCK-2 receptor signaling. This hypothesis was supported by results of real-time RT-PCR, immunofluorescence double labeling and Western blotting assays, which indicated CCK-2 receptor overexpression in PaPo neurons of CCK1R−/− mice. Furthermore, behavioral studies showed that intraperitoneal injections of longlumide up-regulated food accesses in wild-type but not in CCK1R−/− mice, whereas CI-988 injections up-regulated food accesses in CCK1R−/− but not in wild-type mice. Compensatory CCK signaling via CCK-2 receptors in CCK1R−/− mice shed light on currently controversial satiety-controlling mechanisms.

The peptide cholecystokinin (CCK), originally described as a gut hormone (1), is a ubiquitous neuropeptide involved in a variety of homeostatic and physiological functions, but the localization and functions of its receptor are largely unknown. CCK peptides secreted from the gastrointestinal tract in response to ingestion indirectly control hypothalamic satiety centers such as the paraventricular nucleus (PVN) and dorsal medial hypothalamus (DMH), via a large scale afferent network including the vagus nerve and the medullary nucleus of the solitary tract (NTS) (2–5). Of the two subtypes of CCK receptors, CCK-1 receptors (CCK1Rs) are believed to play a pivotal role in satiety control for the following reasons: (i) CCK1Rs are expressed in both the vagus nerve and the hypothalamic satiety centers (5–7); (ii) administration of CCK1R agonists, but not CCK-2 receptor (CCK2R) agonists, reduced ingestion (8, 9); and (iii) larger meal size and obesity were observed in a mutant rat strain, Otsuka Long Evans Tokushima Fatty (OLETF), which lacks CCK1R genes (10, 11). Based on these findings, CCK1R agonists are currently a target for drug development to treat overweight and obesity (12). The principal targets of peripheral CCK for controlling satiety responses may be receptors located in the gut (13, 14) and vagal afferents (15, 16) terminating in the NTS. The NTS may use glutamate as an essential neurotransmitter to convey ascending satiety signals to the brain (17, 18). Within the brain, the PVN and DMH, or other hypothalamic centers involved in food intake behaviors receive projections not only from the NTS but also from neighboring neurons for the integration of various signaling inputs. Intriguingly PVN neurons contain high levels of CCK peptides and show up-regulated transcript-

§ The abbreviations used are: CCK, cholecystokinin; ANOVA, analysis of variance; aCSF, artificial cerebrospinal fluid; [Ca2+], intracellular Ca2+ concentration; CCK1R, CCK-1 receptor; CCK2R, CCK-2 receptor; DMH, dorsal medial hypothalamus; NTS, medullary nucleus of the solitary tract; O.C.T., optimal cutting temperature; OLETF, Otsuka Long Evans Tokushima Fatty; PaPo, posterior part of the hypothalamic paraventricular nucleus; PVN, paraventricular nucleus; TTX, tetrodotoxin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; TBS-T, Tris-buffered saline-Tween 20; CCK-8s, CCK-8 sulfate.

*This work was supported in part by a Grant-in-Aid for Scientific Research (223000108) from the Ministry of Education, Culture, Sports, Science, and Technology Japan (to M. I.).

‡ Both authors contributed equally to this work.

§ To whom correspondence should be addressed; Dept. of Biology, Faculty of Science, University of Toyama, Rm. B214, 3190 Gofuku, Toyama-city, Toyama 930-8555, Japan. Tel.: 81-76-445-6636; Fax: 81-76-445-6549; E-mail: msikedata@sci.u-toyama.ac.jp.
Synergic Control of Satiety via Two CCK Receptor Subtypes

tion of CCK1R and -2R genes in response to osmotic stress (19). In addition, the hypothalamic suprachiasmatic nucleus, the center of the biological clock, contains CCK peptides (20) and sends axons to the PVN and DMH (21). Therefore, PVN circuits, including CCK-mediated satiety control mechanisms, may undergo circadian clock regulation. Centrally administered CCK was sufficient to produce a satiety response and this response was mediated by CCK1Rs but not CCK2Rs (22, 23). Therefore, analysis of the localization and functions of CCK1Rs in the brain is important for a complete understanding of satiety systems.

A previous study of ours visualized CCK1R gene expression in several hypothalamic nuclei involved in food intake behaviors, including the PVN, DMH, ventral medial hypothalamus, and arcuate nuclei, with the highest level of expression being found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent c-Fos and oxytocin gene expressions (24) and fasting-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7). Satiety-dependent estrogen receptor immunoreactivities (25) were found in the posterior subregion of PVN (PaPo) (7).

**EXPERIMENTAL PROCEDURES**

**Mice**—Mice bred under a light-dark cycle (lights on 08:00–20:00) at a constant temperature (22 ± 1 °C) were used in all experiments. Male CCK1R−/− mice and their wild-type control littermates were generated as described previously (7, 27). Experiments were approved by the Committee of Animal Care of University of Toyama and National Kyushu Cancer Center.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) Staining—2–3-Month-old male CCK1R+/− mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS) for 5 min followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min. Whole brains were removed and further fixed in the same fixative (4 °C, overnight). Frozen sections of 50-μm thickness were cut using a cryostat microtome and washed three times with PBS, after which they were mounted on glass slides. The samples were then stained for 7 h at 37 °C using an X-gal staining kit (K1465-01, Invitrogen) according to the manufacturer's instructions. Further details of X-gal staining techniques have been described previously (7).

Immunostaining for β-Galactosidase—Brain sections (30-μm thickness) prepared as described above were used for immunofluorescence staining. Fixed sections were rinsed three times with PBS and then incubated in blocking solutions containing 10% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.1% Triton X-100 (Sigma) for 24 h at 4 °C. Then, samples were incubated with 1:2000 mouse anti-β-galactosidase (Sigma) dissolved in PBS containing 5% donkey serum and 0.05% Triton X-100 for 48 h at 4 °C. After three 15-min PBS rinses, samples were incubated in 1:200 Cy3-conjugated donkey anti-mouse IgG (Jackson). After five 20-min PBS rinses, samples were incubated with 1:1000 4,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kumamoto, Japan) dissolved in PBS for 20 min at room temperature (22–26 °C). These samples were thoroughly washed with PBS and then embedded with Elvanol polyvinyl alcohol. Fluorescence images were acquired using a cooled charge-coupled device (CCD) camera (Ds-2MBWc, Nikon, Tokyo, Japan), 100-W xenon lamp, and standard FITC/Cy3 filter sets mounted on an inverted microscope (TE-2000, Nikon). Digital images were processed using Photoshop 6.0 software (Adobe Systems, San Jose, CA).

Immunostaining for CCK Receptors—Hypothalamic slice cultures were prepared as described previously (28) and used for two-step double immunofluorescence staining for CCK1Rs and -2Rs. The staining was performed using antibodies against CCK1Rs (CCK-AR (N-20) antibody, sc-16172, Santa Cruz Biotechnology, Santa Cruz, CA) and CCK2Rs (gastrin receptor polyclonal antibody, bs-1777R, Bioss, MA). Slices were fixed with 4% paraformaldehyde in PBS at pH 7.4 for 5 min. The samples were rinsed three times with PBS and incubated in blocking solutions containing 10% donkey serum and 0.05% Triton X-100 for 24 h at 4 °C to block nonspecific antibody binding. Samples were then incubated for 48 h at 4 °C with 1:100 rabbit anti-CCK2R antibody dissolved in PBS containing 5% donkey serum. After three 15-min PBS rinses, the samples were incubated with 1:200 Cy3-conjugated donkey anti-rabbit IgG antibody overnight (<20 h) at 4 °C. Following five 20-min rinses with PBS, the sample was incubated with 1:200 goat anti-CCK1R antibody dissolved in PBS containing 5% donkey serum. After three 15-min PBS rinses, the samples were incubated with 1:200 Alexa 647-conjugated donkey anti-goat IgG (Jackson) antibody overnight at 4 °C. Following five 20-min rinses with PBS, samples were incubated with 1:1000 DAPI solution. The samples were thoroughly washed with PBS and then embedded with Elvanol polyvinyl alcohol. Immunofluorescence images were viewed using a confocal imaging system equipped with an inverted microscope, UPLSAPO ×60 NA1.35 oil immersion objective lens, and argon/helium neon lasers (Fluoview 1000, Olympus, Tokyo, Japan). Scanning parameters were unified across specimens.

Ca2+ Imaging in Acutely Isolated Brain Slices—Coronal hypothalamic slices containing the PVN were prepared, stained by fura-2-AM (Dojindo Laboratories), and observed using an upright microscope (Axioplan2; Carl Zeiss, Thornwood, NY) with a water-immersion objective as described previously (29). During recordings, slices were placed in a 0.5-ml bath chamber and perfused with artificial cerebrospinal fluid (aCSF) at a flow rate of 2.5 ml/min. Unless otherwise noted, the aCSF used in experiments contained tetrodotoxin (TTX; 0.5 μM, Sankyo Co., Tokyo, Japan), CCK-8s (Sigma), Iorglumide (LGM; LKT Laboratories Inc., St. Paul, MN), thapsigargin (Sigma), CCK-4 (Pep
Synergic Control of Satiety via Two CCK Receptor Subtypes

tide Institute, Osaka, Japan), CI-988 (Tocris, Bristol, UK), and glutamate (Wako Pure Chemical Industries, Osaka, Japan) were applied by switching the perfusate. Glutamate stimulation was used as a positive control for neuronal responses because about 75% of PVN cells with neuronal cell shapes (excluding larger astroglial cells) showed increased levels of Ca\(^{2+}\) following bath application of 100 \(\mu\)m glutamate.

**Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay**—Male wild-type and CCK1R\(^{-/-}\) mice aged 2 months were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Whole brains were then removed and directly frozen on dry ice. Frozen brains were transferred to a cryostat chamber at \(-30^\circ\)C, mounted with O.C.T. compound (Sakura Finetek, Tokyo, Japan) at \(-15^\circ\)C and sectioned at a thickness of 100 \(\mu\)m. Frozen sections were transferred onto glass slides, punched out using flat-top stainless pipettes (inner diameter, 0.33 mm; hand-made from 23-gauge disposable syringe tips) on ice and homogenized using a disposable microhomogenizer (Bio-masher, Funakoshi, Tokyo) with 600 \(\mu\)l of buffer RLT (RNeasy kit, Qiagen, Chatsworth, CA) at 2,500 \(\times\) g for 30 s. Following the addition of 600 \(\mu\)l of 70% ethanol, samples were stored at \(-80^\circ\)C. Total RNA (4 \(\mu\)g/sample) was extracted from tissue homogenates using an RNeasy kit according to the manufacturer’s instructions. Reverse transcription, including DNase treatment, was performed using a QuantiTect reverse transcription kit (Qiagen) with standard procedures. PCR primers were as follows: CCK1R forward primer, GACAGCCTTTCTATGATGG-GAG; CCK1R reverse primer, GCTGAGGTGATCCAGCCAGCAG; CCK2R forward primer, GATGGCCTGCTACTGCACT; CCK2R reverse primer, GCCACACCCGTCTTCTTGA; \(\beta\)-actin forward primer, AGTGTGACGTGACATCCGTGA; \(\beta\)-actin reverse primer, GCCAGAGCATTAATCCTTC. Real-time PCR was performed using the Rotor Gene 3000A system (Corbett Research, Mortlake, NSW, Australia) with a 72-well rotor. PCR consisted of template cDNA, 2\(\times\) Rotor-Gene SYBR Green, forward and reverse primers (50 \(\mu\)m, each), and RNase-free water in a 0.1-ml strip tube, and underwent the following cycling conditions: initial PCR activation was at 95 \(^\circ\)C for 5 min followed by 60 cycles of 95 \(^\circ\)C for 5 s and 60 \(^\circ\)C for 10 s. Reactions in four separate tubes were averaged for each sample. The amount of gene product in each sample was determined by the comparative quantification method using Rotor Gene 6.0 software (Corbett Research). The amount of gene product for the gene of interest was expressed relative to that of \(\beta\)-actin to normalize for differences in total cDNA between samples.

**Western Blotting**—Wild-type and CCK1R\(^{-/-}\) mice aged 2 months were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Whole brains were removed and sliced as described above for Ca\(^{2+}\) imaging analysis. Hypothalamic nuclei were trimmed on ice and homogenized in 1:100 ratio of protease inhibitor mixture (Sigma) and cell lysis solution (50 mm Tris-HCl, pH 7.5, 0.15 mm NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) using a bio-masher (Funakoshi). Microsomal proteins were spun down by centrifugation at 15,000 \(\times\) g for 2 \(\times\) 20 min at 4 \(^\circ\)C. The supernatants were used for standard Western blotting assays. Proteins were resolved by SDS-PAGE (10% acrylamide Mini-PRO-TEAN TGX Gel; Bio-Rad) and electroblotted onto 0.45-\(\mu\)m nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% Block-Ace (DS Pharma Biomedical, Osaka, Japan) in detergent-supplemented Tris-buffered saline (TBS-T; 20 mm Tris, 150 mm NaCl, 0.05% Tween 20, pH 7.5). Membranes were subjected to rabbit polyclonal CCK2R antibody (1:200) (Santa Cruz Biotechnology) and rabbit polyclonal GAPDH antibody (1:400) (Santa Cruz Biotechnology) in TBS-T overnight at 4 \(^\circ\)C, washed in TBS-T (5 \(\times\) 10 min), then incubated for 1 h with HRP-conjugated donkey anti-rabbit IgG (1:50,000) (Jackson) in TBS-T. After extensive washing (6 \(\times\) 10 min) with TBS-T using a rotor shaker, membranes were subjected to luminol reactions using standard procedures (Immun-Star Western C kit; Bio-Rad laboratories). Luminescence intensity was quantified using a monochromatic cooled CCD system installed in a black box (E-Z capture II; Atto Biotechnology, Japan).

**Behavioral Analysis**—Male wild-type mice and CCK1R\(^{-/-}\) mice aged 2 months were individually housed in originally designed acrylic chambers (H35 \(\times\) W17 \(\times\) D25 cm) in which food access was detected by a touch sensor (PS-306, Elekite, Fukuoka, Japan). The sensing probe was connected to a standard stainless pellet server. Because the bottom of the pellet server was placed 5 cm above nesting chips, purposeful food access behaviors, but not general locomotor activities, were counted using this system. The on/off signals from the sensor were fed into a laptop computer through a photo-coupler isolated digital I/O card (PIO-16/16L, Contec Inc., Tokyo, Japan) and automatically counted at 3-min intervals by software written by one of us (M. I.). To analyze the effects of CCK receptor antagonists on food intake behaviors, wild-type (\(n = 14\)) and CCK1R\(^{-/-}\) (\(n = 14\)) mice were individually reared in the recording cages under standard light-dark cycles (lights on 08:00–20:00) and received daily intraperitoneal injections of sterilized saline (90 \(\mu\)l) at 19:30–20:00 for 5 days. Subsequently, the injectant was replaced with 5% dimethyl sulfoxide in sterilized saline (vehicle) on the sixth day and CI-988 (2 mg/kg, dissolved in the vehicle) or LGM (2 mg/kg, dissolved in the vehicle) on the seventh day.

**Statistical Analysis**—Data are presented as mean \(\pm\) S.E. One-way analysis of variance (ANOVA) followed by Duncan’s multiple range tests were used for statistical comparisons across multiple means. Two-tailed Student’s \(t\) tests were used for pairwise comparisons. A 95% confidence level was considered to indicate statistical significance.

**RESULTS**

**Localization of CCK1R Gene Expression in the Hypothalamic PVN**—To visualize the localization of CCK1Rs in the PVN, the present study used X-gal staining and \(\beta\)-galactosidase immunostaining of a series of hypothalamic sections prepared from CCK1R\(^{+/+}\) mice (Fig. 1). Within the sections analyzed, the PaPo displayed higher staining intensities than the anterior-to-medial PVN or other nuclei involved in satiety control (Fig. 1A). The total number of immunoreactive cells counted within the approximate PVN boundaries in posterior sections (including PaPo) was 3.3 times higher than that in the medial PVN and 9.3 times higher than that in the anterior PVN (\(F_{2,6} = 349, p < 0.01;\))
Synergic Control of Satiety via Two CCK Receptor Subtypes

Fig. 1C). This difference did not depend on cell density because the highest degree of counterstaining was observed in the medial PVN (Fig. 1B). The cell population ratio for β-galactosidase immunoreactivity in the posterior PVN (28.4 ± 0.6%) was 6–7 times higher than that in the anterior to medial PVN (F₀.₆ = 641, p < 0.01; Fig. 1D).

Localization of CCK1R-mediated Intracellular Ca²⁺ Mobilization in the Hypothalamic PVN—To further visualize functional CCK1R distributions in the PVN, the present study examined fura-2-based Ca²⁺ imaging experiments for acutely isolated hypothalamic slices. The PVN slices were stimulated briefly with 10 nM CCK-8s under circulation of aCSF. Most of the PVN cells showed increased intracellular Ca²⁺ concentration ([Ca²⁺]₀(33 of 81 cells in 4 slices; 40.3 ± 2.3%), with a few exceptions that showed decreased [Ca²⁺]₀ (3 of 81 cells in 4 slices; 3.6 ± 2.3%). The inhibitory action of CCK-8s was completely blocked by addition of TTX to aCSF, indicating an indirect response via inhibitory inter-neurons. Therefore, later studies aiming to analyze local Ca²⁺ responses were conducted using TTX supplementation.

Within sequential hypothalamic slices, cells showing CCK-8s (10 nM)-induced Ca²⁺ level increases were located preferentially in the posterior PVN (Fig. 2A). The CCK-8s (10 nM)-induced Ca²⁺ response was triggered by CCK1Rs because the response was negligible in slices prepared from CCK1R−/− mice (Fig. 2A).

Genotype, Dose, and Time of Day-dependent Ca²⁺ Increase via CCK-8s in PaPo Cells—The dose-response of the CCK-8s-induced Ca²⁺ rise was studied in PaPo cells prepared from wild-type and CCK1R−/− mice. The CCK1R−/− cells displayed no apparent Ca²⁺ elevations at 10 nM, but did display a Ca²⁺ level increase similar to that in wild-type cells at 100 nM (Fig. 2B). Accordingly, the EC₅₀ was estimated at 15.2 nM for wild-type cells and at 48.7 nM for CCK1R−/− cells (Fig. 2C).

Day-night variations in responsive cell populations were also analyzed in wild-type slices. Larger numbers of cells responded to 10 nM CCK-8s during the nighttime than during the daytime (+8.8%, p < 0.05; Fig. 2D). However, no day-night differences were observed in the responses to 100 nM CCK-8s or glutamate. The 10 nM CCK-8s stimulation in wild-type slices and the 100 nM CCK-8s stimulation in CCK1R−/− slices evoked Ca²⁺ release from internal Ca²⁺ stores because Ca²⁺ mobilizations following these stimuli were resistant to extracellular Ca²⁺-free buffer but were abolished following depletion of internal Ca²⁺ stores by 10 μM thapsigargin (Fig. 3).

Synergic Ca²⁺ Mobilizations between CCK1R and -2R in PaPo Neurons—To further characterize the mechanisms underlying CCK-8s-induced Ca²⁺ responses in PaPo cells, we examined the effects of CCK antagonists on these responses. First, wild-type PaPo cells were repeatedly stimulated with CCK-8s (10 nM) or CCK-4 (100 nM), a specific agonist for CCK2Rs (Figs. 4, A and B). The CCK-8s (10 nM)-induced Ca²⁺ transients were strongly blocked by LGM (10 μM), a CCK1R antagonist (Fig. 4A, middle). CCK-4 (100 nM) similarly evoked Ca²⁺ transients in PaPo cells (respective cell populations = 41.3 ± 3% in 4 slices) and this response was completely blocked by CI-988, a CCK2R antagonist (Fig. 4B). These results denote CCK1R and -2R functions in the PaPo and theoretical antagonistic activities of LGM and CI-988 for these receptors.

Subsequently, repeated stimulations by a high concentration of CCK-8s (100 nM) were examined in the presence of LGM and CI-988 (Fig. 4, C and D). In wild-type slices, the 100 nM CCK-8s-induced Ca²⁺ transients were partially blocked by 10 μM LGM (Fig. 4C, middle) or 1 μM CI-988 (Fig. 4C, right), whereas a mixture of these antagonists completely blocked the Ca²⁺ transients (Fig. 4C, left). On the other hand, the 100 nM CCK-8s-induced Ca²⁺ transients in CCK1R−/− slices were almost completely blocked by CI-988 alone (Fig. 4D, right), whereas
Synergic Control of Satiety via Two CCK Receptor Subtypes

FIGURE 2. Intracellular Ca\(^{2+}\) mobilizations in the hypothalamic PVN. A, the distribution of PVN cells showing a CCK-8s (10 nM)-induced Ca\(^{2+}\) increase under TTX perfusion was analyzed using sequential PVN slices. Slices from CCK1R\(^{-/-}\) mice (-/-) did not show CCK-8s (10 nM)-induced Ca\(^{2+}\) increases in any PVN subregion. There was a significantly larger responsive cell population in the posterior part of PVN as compared with the anterior or medial regions in wild-type slices (+/+). Six slices were used for each group. **, F\(_{6,21}\) = 63.41, p < 0.01 as compared with the wild-type anterior PVN and #, F\(_{6,21}\) = 63.41, p < 0.01 as compared with the wild-type medial PVN by Duncan’s multiple range test following one-way ANOVA. B, posterior PVN cells were repeatedly stimulated by different concentrations of CCK-8s to analyze dose responses. Representative cell responses in wild-type (+/+), heterozygous (+/-), and CCK1R\(^{-/-}\) (-/-) mice are shown. Note that CCK-8s (10 nM) increased intracellular Ca\(^{2+}\) levels in wild-type and heterozygous slices, but not in CCK1R\(^{-/-}\) slices. All experiments were conducted in the presence of 0.5 \(\mu\)M TTX. Glutamic acid was applied at the end of experiments as a positive control. C, dose-response curve for CCK-8s showing Ca\(^{2+}\) responsiveness. EC\(_{50}\) was estimated at 15.2 nM for wild-type cells and 48.7 nM for CCK1R\(^{-/-}\) cells. D, responsive cell populations in wild-type PaPo slices were compared between daytime and nighttime. Six slices were used for each population analysis. *p < 0.05 by Student’s t test.

LGM failed to block the responses (Fig. 4D, middle). The mean amplitude of Ca\(^{2+}\) response caused by 100 nM CCK-8s in CCK1R\(^{-/-}\) slices reached 91% of that in wild-type slices although the amplitude is statistically different (\(\Delta[Ca^{2+}]\)i = 0.089 ± 0.001 for wild-type and \(\Delta[Ca^{2+}]\)i = 0.081 ± 0.001 for CCK1R\(^{-/-}\) slices, p < 0.01; Fig. 4, C and D). In addition, the mean amplitude of Ca\(^{2+}\) response caused by 100 nM CCK-8s in CCK1R\(^{-/-}\) slices was 33% higher than that caused by 100 nM CCK-4 in wild-type slices (\(\Delta[Ca^{2+}]\)i = 0.061 ± 0.001, number of cells = 126; p < 0.01; Fig. 4, B and D).

Consequently, dose-response curves for CCK-8s-induced Ca\(^{2+}\) mobilizations were re-analyzed for PaPo cells under LGM or CI-988 treatments (Fig. 5). In the wild-type cells, CCK-8s the EC\(_{50}\) value under LGM treatment (i.e. net CCK2R response; 55.4 nM) was 4.5-fold that under CI-988 treatments (net CCK1R response; 12.2 nM). By CCK1R\(^{-/-}\), CCK-8s EC\(_{50}\) value under LGM treatment in CCK1R\(^{-/-}\) cells was slightly reduced (0.78-fold wild-type level; 43.2 nM) and the maximal amplitude was significantly amplified (1.74-fold wild-type level). These results indicate elevated affinity and efficacy of CCK2Rs by CCK1R\(^{-/-}\).

Compensatory CCK2R Gene Expression in CCK1R\(^{-/-}\) Mice—The transcriptional activities of genes encoding CCK1R and -2R were studied using real-time RT-PCR on tissue punchouts of PVN subregions and other brain regions regulating satiety. In the wild-type PVN, the relative abundance of CCK1R mRNA in the posterior PVN was 3.7 times that in the anterior PVN and 1.6 times that in the medial PVN (F\(_{2,9}\) = 6.0, p < 0.01; Fig. 6A). In wild-type brains, the level of CCK1R mRNA expression in the posterior PVN was largest among those in other regions analyzed in the present study (F\(_{5,21}\) = 6.1, p < 0.01; Fig. 6A and B). On the other hand, CCK2R mRNA was ubiquitous and showed no statistically significant differences in abundance among wild-type PVN subregions (F\(_{2,9}\) = 1.7, N.S.). Notably, the level of CCK2R mRNA expression in CCK1R\(^{-/-}\) PVN sub-
FIGURE 4. Pharmacological blockage of CCK-induced Ca\(^{2+}\) mobilizations in PaPo cells. A, left, CCK-8s (10 nM)-induced Ca\(^{2+}\) mobilization in wild-type PaPo cells was reproducible at 25-min gaps. The second Ca\(^{2+}\) rises were blocked by a CCK1R antagonist (LGM, 10 \(\mu\)M; middle) but were resistant to a CCK2R antagonist (CI-988, 1 \(\mu\)M; right). B, left, the specific CCK2R agonist CCK-4 (100 nM) also evoked a reproducible Ca\(^{2+}\) rise in wild-type PaPo cells at 25-min gaps. The second Ca\(^{2+}\) rises were blocked by CI-988 (1 \(\mu\)M; right) but were resistant to LGM (10 \(\mu\)M; middle). C, CCK-8s (100 nM)-induced Ca\(^{2+}\) mobilization in wild-type PaPo cells were reproducible at 25-min gaps (gray traces on left). The second Ca\(^{2+}\) rises were partially blocked by 10 \(\mu\)M LGM (middle) or by 1 \(\mu\)M CI-988 (right) but completely blocked by a mixture of the two (black traces on left). D, left, similar repeated Ca\(^{2+}\) mobilizations were produced by CCK-8s (100 nM) stimulation in the PaPo cells of CCK1R\(^{-/-}\) mice (−/−). However, the second Ca\(^{2+}\) rises were resistant to LGM (10 \(\mu\)M; middle) but were abolished by CI-988 (1 \(\mu\)M, right). These results indicate that a synergic contribution of CCK1Rs and -2Rs co-expressed in PaPo neurons determines CCK-induced Ca\(^{2+}\) mobilization in PaPo cells. All above responses were repeatedly observed in at least 30 cells in four slices. Of these, representative responses in three cells are plotted.
regions were twice as abundant compared with the corresponding wild-type subregions (Fig. 6A). Also, the level of CCK2R mRNA expression in CCK1R−/− NTS was twice as abundant compared with wild-type NTS (Fig. 6B). There was a tendency for CCK2R mRNA levels to increase in CCK1R−/− mice in the ventromedial hypothalamus (1.5 times wild-type levels) and the arcuate nucleus (1.4 times wild-type levels), but these differences were not statistically significant (Fig. 6B). These results indicate the existence of compensatory CCK2R gene expression in CCK1R−/− mice depending on brain regions.

**Co-expression and Compensatory Expression of CCK1R and CCK2R Proteins in PVN Neurons**—The above series of evidence denotes compensatory expression and functional synergy between CCK1R and -2R in PVN neurons; we confirmed this by immunofluorescence staining and Western blotting studies. Because visualization of plasma membrane receptor immunoreactions in cryostat sections was difficult, we used organotypic slice cultures in which live neurons were located on thin slice surfaces to visualize cellular localization of receptor proteins. Within the PVN, the CCK1R antibody successfully recognized the subpopulation of parvocellular PVN neurons where signals were found both in cell bodies and dendrites (Fig. 7A). CCK2R antibody recognized much larger cell populations in the PVN (Fig. 7A). Most CCK1R-immunoreactive (-ir) cells overlapped with CCK2R-ir cells (62 of 64 cells in four slices), demonstrating co-localization of CCK1R and -2R in PVN neurons. Consistent with the transcriptional levels, Western blotting analysis clearly demonstrated up-regulation of CCK2R protein levels in the PVN of CCK1R−/− mice (2.4-fold wild-type levels; p < 0.01; Fig. 7B). Nevertheless, the number of CCK2R-ir cells was not increased by CCK1R−/− (cell density = 30.8 ± 1.5/field for the wild-type and 32.3 ± 2.5/field for CCK1R−/−, Fig. 7A). This indicates that compensatory expression of CCK2R occurred in cells that originally express CCK2R, including CCK1R-positive cells, although the CCK2R-ir intensity was equalized by image processing using confocal scanning microscopy.

**Functional Compensation between CCK1Rs and -2Rs for Satiety Control**—To analyze whether functional compensation between the two CCK receptor subtypes could have an influence at a system level, the effects of CCK antagonists on food access behaviors were monitored in wild-type and CCK1R−/− mice. Wild-type and CCK1R−/− mice displayed similar food intake behaviors: high during the night and low during the day (Fig. 8, A and B). The intraperitoneal injection of LGM (2 mg/kg) at the onset of darkness facilitated successive nighttime food accesses only in wild-type mice (+11.1% of vehicle injected controls; F_{3,25} = 4.9, p < 0.01) but not in CCK1R−/− mice (+3.8% of vehicle-injected controls; N.S.; Fig. 8C). On the other hand, intraperitoneal injection of CI-988 (2 mg/kg) at the onset of dark facilitated successive nighttime food accesses only in CCK1R−/− mice (+15.0% of vehicle injected controls; F_{3,20} = 3.4, p < 0.05), but not in wild-type mice (+0.8% of vehicle-injected controls; N.S.; Fig. 8D). These results indicate that CCK1R signaling for satiety control switched to CCK2R signaling in CCK1R−/− mice.
DISCUSSION

The present study analyzed the functions of the CCK receptor in mouse hypothalamic slices using Ca²⁺ imaging techniques and demonstrated significant CCK receptor functions in the PaPo. Localized CCK-8s-induced Ca²⁺ responses are consistent with reporter gene identification of CCK1R expression. In PaPo neurons, both CCK1Rs and -2Rs may be involved in endogenous CCK signaling, because the synergic contributions of CCK1Rs and -2Rs were observed for CCK-8s (≥30 nM)-induced Ca²⁺ mobilizations. The substitutive CCK2R gene/protein expression revealed by real-time RT-PCR and Western blotting explains the compensatory CCK2R functions in the PaPo of CCK1R⁻/⁻ mice. CI-988 injection activated food access behaviors in CCK1R⁻/⁻ mice, but not in wild-type mice, further suggesting the functional compensation between CCK1Rs and -2Rs at a system level. It has been widely shown that pharmacological blockage of CCK1Rs reduces satiety and facilitates feeding, and CCK1R agonists are candidate drugs to treat overweight and obesity. On the other hand, conventional gene knock-out mice lacking CCK signaling do not always follow this pharmacological evidence, resulting in controversial

FIGURE 7. A, the distribution of CCK1R and -2R proteins in the PaPo was analyzed by immunofluorescence double labeling and confocal microscopy. CCK1R (green), CCK2R (red), and DAPI nuclear staining (blue) were superimposed on the merged color images. Note that most of the CCK1R signals overlapped the CCK2R signals, resulting in the yellow color in the CCK1R-positive cells in the wild-type slice (+/+) Negligible staining levels for CCK1R were found in the CCK1R⁻/⁻ slice (−/−). B, CCK2R protein abundance in the hypothalamus was further quantified using Western blotting. Relative abundance of CCK2R protein in PVN was larger in the CCK1R⁻/⁻ mice (−/−) than in the wild-type mice (+/+) Corresponding differences were not detectable in the DMH or in any of the VMH and ARC regions. **, p < 0.01 by Student’s t test. Four samples were used to calculate the average.

FIGURE 8. A, injection of LGM (2 mg/kg intraperitoneal at dark onset) up-regulated food access in wild-type (+/+) but not in CCK1R⁻/⁻ mice (−/−). The gray area in the plots denotes the number of food accesses after vehicle injection 1 day before LGM injection. The black area in the plots denotes the number of food accesses after LGM injection. n = 8 for each genotype. B, injection of CI-988 (2 mg/kg, intraperitoneal at dark onset) increased food accesses in CCK1R⁻/⁻ mice (−/−) but not in wild-type mice (+/+). Gray and black areas denote vehicle and CI-988 injected groups, respectively. n = 6 for each genotype. *, p < 0.05 by Student’s t test in comparison with the corresponding vehicle-injected controls. Black and white bars on the bottom denote light and dark periods. C, the 12-h cumulative number of food accesses following the LGM injection was calculated for further comparisons between genotypes. The 12-h cumulative number of food accesses following injection of CI-988 injection was also analyzed. *, p < 0.05; **, p < 0.01 by one-way repeated ANOVA. Dotted lines denote the 12-h cumulative numbers of food accesses in vehicle-injected wild-type mice, which were equivalent to those in vehicle-injected CCK1R⁻/⁻ mice.
interpretations in satiety-controlling mechanisms. Thus, the functional compensation between CCK1Rs and -2Rs observed in the present study explains this inconsistency and sheds light on the mechanisms underlying CCK-mediated satiety control.

The PaPo as a Center for CCK1R-mediated Autonomic Controls—The hypothalamic PVN is an important integrating site for autonomic and endocrine functions (30). This nucleus is a heterogeneous neuronal cluster consisting of magnocellular and parvocellular neurons that are largely segregated into specific anatomical compartments (31, 32). The parvocellular PVN cells are further categorized into different neuronal types, including neuroendocrine neurons that project to the median eminence and regulate the release of hormones from the anterior pituitary gland, and pre-autonomic neurons that send long descending projections to the brainstorm and spinal cord regions that are important with respect to autonomic control. The PaPo cells have been categorized as parvocellular pre-autonomic PVN cells (33). A recent study demonstrated that the PaPo is the locus of satiety-dependent c-Fos and oxytocin gene expression (24), but the neuronal circuits underlying satiety controls in the PaPo have not been clearly demonstrated.

We previously examined X-gal staining of broad hypothalamic areas of CCK1R+/− mice and demonstrated high staining intensity in the hypothalamic PVN, particularly in the PaPo (7). The present study further analyzed the detailed distribution of CCK1Rs in hypothalamic PVN and demonstrated significant CCK1R density in PaPo in comparison to the anterior to medial PVN. The gradient in CCK1R expression levels within the PVN was also supported by the results of a real-time RT-PCR assay. Furthermore, Ca2+ imaging experiments reveal a distribution of functional CCK1Rs that is consistent with gene expression profiles. Koutcherov et al. (34) analyzed the structure of human hypothalamic PVN using immunohistochemistry and demonstrated significant levels of acetylcholine esterase expression in the PaPo. Consistently, our preliminary observations indicate that about half of PaPo cells were carbachol responsive. However, carbachol responses rarely overlapped CCK-8s responses in the PaPo (data not shown), suggesting that cholinergic and CCKergic cells are neighboring but distinct cell populations. Intravenous administration of CCK-8s elevated c-Fos expression in the oxytocin-secreting parvocellular neurons in the PVN and this response was absent in OLETF rats (35). Therefore, oxytocin-positive but not cholinergic PaPo cells possibly play a pivotal role in CCK-mediated satiety control in the PVN.

Intracellular Ca2+ Mobilizations via Synergic Activation of CCK1Rs and -2Rs in PaPo Neurons—CCK-8s is a general CCK receptor agonist with twice the affinity for CCK1Rs than for CCK2Rs (12). CCK-8s is sometimes described as a specific CCK1R agonist, even when used at high concentrations (100–1,000 nM) on in vitro slices (36). However, the present study carefully analyzed the dose–response curves both for wild-type and CCK1R+/− slices and showed that only a low concentration (10 nM) CCK-8s specifically mobilized Ca2+ via CCK1Rs. Concentrations higher than 30 nM mobilized Ca2+ both via CCK1Rs and -2Rs in PaPo cells.

If CCK1Rs and -2Rs are separately expressed in different PaPo cell populations, LGM or CI-988 might inhibit the CCK-8s (>30 nM)-induced Ca2+ response dependent on the focused cell. However, the present results showing that the Ca2+ response was partially inhibited by LGM or CI-988 and completely abolished by a co-mixture of LGM and CI-988 demonstrate co-expression of these two receptor subtypes in single PaPo neurons. This hypothesis is strongly supported by the results of immunofluorescence staining of receptor proteins, by which overlapping expression of CCK1R and -2R in PaPo neurons was observed.

CCK1Rs and -2Rs have a high degree of structural homology and both are G protein-coupled receptors linked to the phosphatidylinositol cascade to mobilize Ca2+ from endoplasmic reticulum Ca2+ stores following their activation (37, 38). Consistent with the theoretical signaling reported for model cells, we observed Ca2+ mobilizations from internal Ca2+ stores via both CCK1Rs and -2Rs (i.e. low and high concentration CCK-8s) in PaPo cells. It has been shown that CCK1Rs and -2Rs form heterodimers that enhance signaling in CHO cell models (39). Whether such synergic and direct interactions between these two receptor subtypes could occur in a real biological system was unknown. The present study revealed co-localization of CCK1Rs and -2Rs in identical PaPo neurons using immunofluorescence double labeling. It also showed Ca2+ mobilizations via CCK1Rs and -2Rs in identical PaPo neurons in wild-type slices. The amplitudes of Ca2+ transients caused by co-stimulation of CCK1Rs and -2Rs were significantly larger than those caused by single subtype stimulation. This additive action was also seen in CHO cells that received double transfection with CCK1R and -2R genes (39). Therefore, we assume that there is a synergic contribution of these two receptors to CCK-mediated Ca2+ signaling in PaPo cells, at least via pharmacological stimulation with CCK-8s.

Ablated CCK1R Functions Were Compensated by CCK2R Functions in CCK1R+/− Mice—The redundant CCK1R and -2R signaling in PaPo cells raised questions as to whether the co-stimulation of two receptor subtypes is essential for endogenous CCK-mediated satiety controls. Because Gibbs et al. (40) characterized CCK as a satiety factor and two CCK receptors were successfully cloned (41, 42), specific CCK receptor ligands to control satiety have been widely explored. It has been shown that ligands for CCK1Rs rather than those for CCK2Rs can successfully control satiety (see Refs. 8 and 9; also in Fig. 8). Therefore, it seems likely that there is a preferential or larger contribution of CCK1R-mediated signaling to endogenous satiety control. Interestingly, the present study revealed day-night differences in Ca2+ mobilizations via CCK1Rs, whereas no such differences were detected following co-stimulation of CCK1Rs and -2Rs. Thus, intrinsic CCK regulations such as via circadian clock control of the PVN axis (21) may be more closely associated with CCK1Rs. On the other hand, our preliminary experiments indicate functional coupling of CCK2Rs but not -1Rs to leptin signaling in PaPo cells (data not shown), suggesting a possible involvement of CCK2Rs in leptin-mediated satiety controls in a particular physiological situation. Indeed, results showing that antagonists for CCK2Rs but not CCK1Rs micro-infused into the PVN inhibited colonic motility in non-fasted rats (43) suggest a specific function of CCK2Rs in integrative satiety controls via the PVN.
Synergic Control of Satiety via Two CCK Receptor Subtypes

Consistent with the accumulated pharmacological evidence, deletion of CCK1R genes was identified in OLETF rats (10, 11). However, the involvement of CCK1Rs in regulation of satiety is currently controversial. For example, CCK1R<sup>−/−</sup> mice do not develop hyperphagia and obesity when maintained on regular chow (26, 27) despite the obvious obese phenotype of OLETF rats. Bi et al. (5) demonstrated that OLETF rats had elevated neuropeptide Y mRNA expression in the DMH, whereas this was not seen in CCK1R<sup>−/−</sup> mice. Because CCK1Rs are co-localized with neuropeptide Y in the DMH of the control strain rats (5) and knock-down of neuropeptide Y in OLETF rats resulted in a significant reduction in body weight and food intake (44), the phenotypic discrepancy between rats and mice can be explained by differential receptor expression in the DMH and resultant uncoupling from neuropeptide Y signaling. However, Blevins et al. (45) recently demonstrated that CCK1R knock-out rats (F344, Cck1r<sup>−/−</sup>) do not display obesity, similar to CCK1R<sup>−/−</sup> mice; thus, it is also possible that the phenotype of OLETF rats depends on genes other than the CCK1R gene, as shown previously (46).

The present study shows that the functions of deleted CCK1Rs are replaceable by CCK2Rs in CCK1R<sup>−/−</sup> mice, as supported by the following evidence: (i) CCK2R transcription was up-regulated in the PVN and NTS in CCK1R<sup>−/−</sup> mice; (ii) a consistent increase in CCK2R protein levels was found in the PVN of CCK1R<sup>−/−</sup> mice; (iii) δCa<sup>2+</sup>), following CCK2R stimulations was amplified in the PaPo cells of CCK1R<sup>−/−</sup> slices; and (iv) food intake behaviors were up-regulated by CI-988 in CCK1R<sup>−/−</sup> mice. These results show compensatory functions between CCK1Rs and -2Rs and thus may explain the normal satiety controls in CCK1R<sup>−/−</sup> mice. Recently, we observed CCK1R expression in ependymal cells of the third ventricle in early developmental stages, and showed a role for this expression in suckling control of babies (47). As in adult PaPo cells, ependymal cells in the neonatal third ventricle express both CCK1Rs and -2Rs, but functional compensation did not occur during early developmental stages. In fact, CCK1R<sup>−/−</sup> pups displayed an overweight phenotype regardless of maternal genotype. Therefore, functional compensation between CCK1Rs and -2Rs may be processed during postnatal development.

Regular food intake activities and body weights are also reported for CCK null mutant mice (48). Therefore, functional compensation for molecules underlying CCK-mediated satiety controls may not be limited to the two CCK receptor subtypes, but may also include other receptor signaling molecules such as leptin (49, 50). For either case, the results derived from conventional knock-out mice need careful interpretation when considering single molecule functions within a system.

REFERENCES

1. Ivy, A. C., and Oldenberg, E. (1928) A hormone mechanism for gallbladder contraction and evacuation. Am. J. Physiol. 86, 599–613

2. Rinaman, L., Verbalis, J. G., Stricker, E. M., and Hoffman, G. E. (1993) Distribution and neurochemical phenotypes of caudal medullary neurons activated to express cFos following peripheral administration of cholecystokinin. J. Comp. Neurol. 338, 475–490

3. Mönnikes, H., Lauer, G., Bauer, C., Tebbe, J., Zittel, T. T., and Arnold, R. (1997) Pathways of Fos expression in locus ceruleus, dorsal vagal complex, and PVN in response to intestinal lipid. Am. J. Physiol. Regul. Integr. Comp. Physiol. 273, R2059–R2071

4. Reidelberger, R. D., Hernandez, J., Fritzsch, B., and Hulce, M. (2004) Abdominal vagal mediation of the satiety effects of CCK in rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R1005–R1012

5. Bi, S., Scott, K. A., Kopin, A. S., and Moran, T. H. (2004) Differential roles for cholecystokinin a receptors in energy balance in rats and mice. Endocrinology 145, 3873–3880

6. Honda, T., Wada, E., Battey, J. F., and Wank, S. A. (1993) Differential gene expression of CCK(A) and CCK(B) receptors in the rat brain. Mol. Cell. Neurosci. 4, 143–154

7. Shimazoe, T., Morita, M., Ogawa, S., Kojita, T., Goto, J., Kamakura, M., Moriya, T., Shinohara, K., Takiguchi, S., Kono, A., Miyasaka, K., Funakoshi, A., and Ikeda, M. (2008) Cholecystokinin-A receptors regulate photic input pathways to the circadian clock. FASEB J. 22, 1497–1490

8. Moran, T. H., Melegio, P. J., Schwartz, G. J., and McHugh, P. R. (1992) Blockade of type A, not type B, CCK receptors attenuates satiety actions of exogenous and endogenous CCK. Am. J. Physiol. 262, R456–R50

9. Simmons, R. D., Kaiser, F. C., and Hudzik, T. J. (1999) Behavioral effects of AR-R 15849, a highly selective CCK-A agonist. Pharmacol. Biochem. Behav. 62, 549–557

10. Takiguchi, S., Takata, Y., Funakoshi, A., Miyasaka, K., Kataoka, K., Fujimura, Y., Goto, T., and Kono, A. (1997) Disrupted cholecystokinin type-A receptor (CCKAR) gene in OLETF rats. Gene 197, 169–175

11. Moran, T. H., Katz, L. F., Plata-Salaman, C. R., and Schwartz, G. J. (1998) Disordered food intake and obesity in rats lacking cholecystokinin A receptors. Am. J. Physiol. Regul. Integr. Comp. Physiol. 274, R618–R625

12. Berna, M. J., Tapia, A. J., Sancho, V., and Jensen, R. T. (2007) Progress in developing cholecystokinin (CCK)/gastrin receptor ligands that have therapeutic potential. Curr. Opin. Pharmacol. 7, 583–592

13. Raboin, S. J., Reeve, I. R. Jr., Cooper, M. S., Green, G. M., and Sayegh, A. I. (2008) Activation of submucosal but not myenteric plexus of the gastrointestinal tract accompanies reduction of food intake by camostat. Regul. Pept. 150, 73–80

14. Washington, M. C., Murray, C. R., Raboin, S. J., Roberson, A. E., Mansour, M. M., Williams, C. S., and Sayegh, A. I. (2011) Cholecystokinin-B activates myenteric neurons in 21- and 35-day-old but not 4- and 14-day-old rats. Peptides 32, 272–280

15. Smith, G. P., Jerome, C., Cushing, B. J., Eronno, R., and Simansky, K. I. J. (1981) Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. Science 213, 1036–1037

16. Broberger, C., Holmberg, K., Shi, T. I., Dockray, G., and Hökfelt, T. (2001) Expression and regulation of cholecystokinin and cholecystokinin receptors in rat nodose and dorsal root ganglia. Brain Res. 903, 128–140

17. Appleyard, S. M., Marks, D., Koyabashi, K., Okano, H., Low, M. J., and Andresen, M. C. (2007) Visceral afferents directly activate catecholamine neurons in the solitary tract nucleus. J. Neurosci. 27, 13292–13302

18. Guard, D. B., Swartz, T. D., Ritter, R. C., Burns, G. A., and Covasa, M. (2009) NMDA NR2 receptors participate in CCK-induced reduction of food intake and hindbrain neuronal activation. Brain Res. 1266, 37–44

19. Hinks, G. L., Poat, I. A., and Hughes, J. (1995) Changes in hypothalamic cholecystokinin A and cholecystokinin B Receptor subtypes and associated neuropeptide expression in response to salt-stress in the rat and mouse. Neuroscience 68, 765–781

20. van den Pol, A. N., and Tsujimoto, K. L. (1985) Neurotransmitters of the hypothalamic suprachiasmatic nucleus. Immunochemical analysis of 25 neuronal antigens. Neuroscience 15, 1049–1086

21. Aston-Jones, G., Chen, S., Zhu, Y., and Ohinsky, M. L. (2001) A neural circuit for circadian regulation of arousal. Nat. Neurosci. 4, 732–738

22. Crawley, J. N., Fiske, S. M., Durieux, C., Derrien, M., and Roques, B. P. (1991) Centrally administered cholecystokinin suppresses feeding through a peripheral-type receptor mechanism. J. Pharmacol. Exp. Ther. 257, 1076–1080

23. Hirsue, Y., Inui, A., Teranishi, A., Miura, M., Nakajima, M., Okita, M., Nakajima, Y., Himori, N., Baba, S., Kasuga, M. (1993) Cholecystokinin octapeptide analogues suppress food intake via central CCK-A receptors in mice. Am. J. Physiol. 265, R481–R486

24. Uchoa, E. T., Mendes da Silva, L. E., de Castro, M., Antunes-Rodrigues, I., and Elias, L. L. (2009) Hypothalamic oxytocin neurons modulate hypo-
phagic effect induced by adrenalectomy. *Horm. Behav.* **56**, 532–538
25. Panicker, A. K., Mangels, R. A., Powers, J. B., Wade, G. N., and Schneider, J. E. (1998) AP lesion block suppression of estrous behavior, but not estrous cyclicity, in food-deprived Syrian hamsters. *Am. J. Physiol.* **275**, R158–R164
26. Kopin, A. S., Mathes, W. F., McBride, E. W., Nguyen, M., Al-Haider, W., Schmitz, F., Bonner-Weir, S., Kanarek, R., and Beinborn, M. (1999) The cholecystokinin-A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. *J. Clin. Invest.* **103**, 383–391
27. Takiguchi, S., Suzuki, S., Sato, Y., Kanai, S., Miyasaka, K., Jimi, A., Shinozaki, H., Takata, Y., Funakoshi, A., Kono, A., Minowa, O., Kobayashi, T., and Noda, T. (2002) Role of CCK-A receptor for pancreatic function in mice. A study in CCK-A receptor knockout mice. *Pancreas* **24**, 276–283
28. Ikeda, M., Sugiyama, T., Wallace, C. S., Gompf, H. S., Yoshioka, T., Miyawaki, A., and Allen, C. N. (2003) Circadian dynamics of cytosolic and nuclear Ca$^{2+}$ in single suprachiasmatic nucleus neurons. *Neuron* **38**, 253–263
29. Ikeda, M., Yoshioka, T., and Allen, C. N. (2003) Developmental and circadian changes in Ca$^{2+}$ mobilization mediated by GABAA and NMDA receptors in the suprachiasmatic nucleus. *Eur. J. Neurosci.* **17**, 58–70
30. Swanson, L. W., and Sawchenko, P. E. (1980) Paraventricular nucleus. A site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* **31**, 410–417
31. Swanson, L. W., and Kuypers, H. G. (1980) The paraventricular nucleus of the hypothalamus. Cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J. Comp. Neurol.* **194**, 554–570
32. Swanson, L. W., and Sawchenko, P. E. (1983) Hypothalamic integration. Organization of the paraventricular and supraoptic nuclei. *Annu. Rev. Neurosci.* **6**, 269–324
33. Stern, J. E. (2001) Electrophysiological and morphological properties of pre-autonomic neurons in the rat hypothalamic paraventricular nucleus. *J. Physiol.* **537**, 161–177
34. Koutcherov, Y., Mai, J. K., Ashwell, K. W., and Paxinos, G. (2000) Organization of the human paraventricular hypothalamic nucleus. *J. Comp. Neurol.* **423**, 299–318
35. Hashimoto, H., Onaka, T., Kawasaki, M., Chen, L., Mera, Y., Saito, T., Fujihara, H., Sei, H., Morita, Y., and Ueta, Y. (2005) Effects of cholecystokinin (CCK)-8 on hypothalamic oxytocin-secreting neurons in rats lacking CCK-A receptor. *Auton. Neurol.* **121**, 16–25
36. Sorimachi, M., Yamagami, K., and Uramura, K. (2001) Functional expression of cholecystokinin-A receptor on ventromedial hypothalamic neurons in the immature rat brain. *Neurosci. Lett.* **300**, 91–94
37. Kopin, A. S., Lee, Y. M., McBride, E. W., Miller, L. J., Lu, M., Lin, H. Y., Kolakowski, L. F., Jr., and Beinborn, M. (1992) Expression cloning and characterization of the canine parietal cell gastrin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3605–3609
38. Ulrich, C. D., Ferber, I., Holicky, E., Hadac, E., Buehl, G., and Miller, L. J. (1993) Molecular cloning and functional expression of the human gall-bladder cholecystokinin A receptor. *Biochem. Biophys. Res. Commun.* **193**, 204–211
39. Cheng, Z. J., Harikumar, K. G., Holicky, E. L., and Miller, L. J. (2003) Heterodimerization of type A and B cholecystokinin receptors enhance signaling and promote cell growth. *J. Biol. Chem.* **278**, 52972–52979
40. Gibbs, J., Young, R. C., and Smith, G. P. (1973) Cholecystokinin elicits satiety in rats with open gastric fistulas. *Nature* **245**, 323–325
41. Wank, S. A., Harkins, R., Jensen, R. T., Shapira, H., de Weerth, A., and Slattery, T. (1992) Purification, molecular cloning, and functional expression of the cholecystokinin receptor from rat pancreas. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3125–3129
42. Wank, S. A., Pisegna, J. R., and de Weerth, A. (1992) Brain and gastrointestinal cholecystokinin receptor family. Structure and functional expression. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8691–8695
43. Mönikes, H., Tebbe, J., Grote, C., Sonntag, A., Pluntke, K., Sturm, K., and Arnold, R. (2000) Involvement of CCK in the paraventricular nucleus of the hypothalamus in the CNS regulation of colonic motility. *Digestion* **62**, 178–184
44. Yang, L., Scott, K. A., Hyun, J., Tamashiro, K. L., Tray, N., Moran, T. H., and Bi, S. (2009) Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. *J. Neurosci.* **29**, 179–190
45. Blevins, J. E., Overduin, J., Fuller, J. M., Cummings, D. E., Matsumoto, K., and Moralejo, D. H. (2009) Normal feeding and body weight in Fischer 344 rats lacking the cholecystokinin-1 receptor gene. *Brain Res.* **1255**, 98–112
46. Muramatsu, Y., Yamada, T., Taniguchi, Y., Ogino, T., Kose, H., Matsumoto, K., Sasaki, Y. (2005) Pnlip encoding pancreatic lipase is possible candidate for obesity QTL in the OLETF rat. *Biochem. Biophys. Res. Commun.* **331**, 1270–1276
47. Ozaki, T., Moghadam, S., Morioka E., Takigaki, E., and Ikeda, M. (2011) Infant satiety depends on ependymal cholecystokinin-1 receptors. *Soc. Neurosci. Abstr.* **192**, 15
48. Lo, C. M., Samuelson, L. C., Chambers, J. B., King, A., Heiman, J., Jandacek, R. J., Sakai, R. R., Benoit, S. C., Raybould, H. E., Woods, S. C., and Tso, P. (2008) Characterization of mice lacking the gene for cholecystokinin. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R803-R810
49. Wang, L., Barachina, M. D., Martínez, V., Wei, J. Y., and Taché, Y. (2000) Synergistic interaction between CCK and leptin to regulate food intake. *Regul. Pept.* **92**, 79–85
50. Li, Y., Wu, X., Zhou, S., and Owyang, C. (2011) Low-affinity CCK-A receptors are coexpressed with leptin receptors in rat nodose ganglia. Implications for leptin as a regulator of short-term satiety. *Am. J. Physiol. Gastrointest. Liver Physiol.* **300**, G217–G227