Phenotype of neurons in the nucleus of the solitary tract that express
CCK-induced activation of the ERK signaling pathway

Tanja Babic, R. Leigh Townsend, Laurel M. Patterson, Gregory M. Sutton,
Huiyuan Zheng, Hans-Rudolf Berthoud

¹Neurobiology of Nutrition Laboratory, Pennington Biomedical Research Center,
Louisiana State University, Baton Rouge, LA 70808, USA

Short title: CCK activation of NTS neurons

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Correspondence to: Hans-Rudolf Berthoud
Pennington Biomedical Research Center
6400 Perkins Road
Baton Rouge, LA 70808

Phone: (225) 763 2688
Fax: (225) 763 0260
E-mail: berthohr@pbrc.edu
Abstract

The satiating potency of cholecystokinin (CCK) has been well characterized, including its mediation by capsaicin-sensitive vagal primary afferents. We have previously shown that peripherally administered CCK activates the MAPK-signaling cascade in a population of nucleus of the solitary tract (NTS) neurons, and that preventing ERK1/2 phosphorylation partly attenuates CCK’s satiating potency. The aim of this study was to identify the neurochemical phenotypes of the NTS neurons that exhibit CCK-induced activation of ERK1/2. Using confocal microscopy, we demonstrate that intraperitoneal CCK administration increases the number of neurons that express phosphorylated ERK1/2 (pERK1/2) in the medial and commissural subnuclei of the NTS, and that CCK-induced expression of ERK1/2 is increased in tyrosine hydroxylase-immunoreactive neurons. Using Western blotting, we show that the robust increase in tyrosine hydroxylase phosphorylation obtained with ip CCK is significantly attenuated in rats pretreated with the ERK-pathway blocker U0126 injected into the 4th ventricle. In addition, CCK injections increased pERK1/2 expression in POMC neurons in the NTS. In contrast, only the rare GAD67, nNOS, and leptin responsive neuron exhibited CCK-induced pERK immunoreactivity. We conclude that activation of POMC-immunoreactive neurons and tyrosine hydroxylase activity via the ERK-signaling pathway in the NTS likely contributes to CCK’s satiating effects.

Key words: CCK, nucleus of the solitary tract, tyrosine hydroxylase, POMC, leptin receptor, MAPK
Introduction

Increased food intake is an important component of energy balance and a major cause in the development of obesity. Because humans eat relatively few discrete meals, meal size control is a reasonable strategy to limit total food intake (33). Meal size is controlled by the process of satiation and, although considerable progress has been made in identifying the various signals contributing to satiation, the neural mechanisms integrating these signals are poorly understood.

Cholecystokinin (CCK) released from enteroendocrine cells lining the upper small intestine has been generally recognized as the archetypical satiation hormone [for recent review see (11)]. Its signaling pathway via CCK-1 receptor-bearing vagal afferent fibers to the nucleus tractus solitarius (NTS) in the caudal brainstem has been well documented (19, 24, 27, 31, 34, 40). In contrast, it is not clear how activity in the NTS ultimately leads to termination of a meal, and how it is related to a certain period of satiety after the meal. Based on the well-established expression of the immediate-early gene c-Fos in NTS neurons that goes along with the process of satiation, we had hypothesized that these neurons may change their excitability and synthetic machinery to a “satiated” state through changes in intracellular signaling patterns and ultimately changes at the transcription level (7). In support of this hypothesis, we demonstrated that exogenously administered CCK increases activity of the ERK 1/2 → CREB signaling cascade in NTS neurons and that inhibiting this pathway significantly attenuates CCK-induced food intake suppression in rats (35, 36).

The aim of the present study was to identify the neurochemical phenotypes of the NTS neurons exhibiting CCK-induced ERK activation. Our attention was focused on the A2 catecholaminergic neurons located in the NTS (16), because they have been most strongly implicated in CCK-induced satiation. First, a significant percentage of NTS catecholamine neurons express c-Fos upon stimulation with various satiation signals from the gut (29-31, 37). Second, CCK is less able to suppress food intake in rats with selective immunotoxic ablation of NTS catecholaminergic neurons and CCK’s capacity to suppress food intake is proportional to the number of surviving catecholaminergic neurons (28). In addition, tyrosine hydroxylase (TH), a key enzyme in the catecholamine synthetic pathway, has a specific ERK-phosphorylation site at Ser 31(12). Therefore, one aim was to assess the number of catecholaminergic...
neurons exhibiting CCK-induced activation of the ERK pathway and whether CCK is able to activate the ERK-specific phosphorylation site on tyrosine hydroxylase.

Proopiomelanocortin (POMC)-containing neurons in the NTS have also been implicated in mediating satiating effects of CCK (1, 10). Peripheral administration of CCK has been shown to increase the expression of c-Fos in POMC neurons in the NTS and administration of melanocortin receptor antagonist into the fourth ventricle attenuated CCK-induced reduction in food intake (10). However, it is not clear whether activation of POMC neurons in the NTS occurs via activation of the ERK1/2 signaling pathway.

In addition, specific populations of NTS neurons expressing glutamic acid decarboxylase (GAD67), neuronal nitric oxide synthase (nNOS), and leptin receptors may also be involved in the satiation process. Therefore, the second aim was to assess the proportion of these specific neuron populations exhibiting CCK-induced activation of the ERK pathway. Because immunohistochemical identification of POMC, GABA, and leptin receptors is difficult, we used transgenic mouse models expressing green fluorescent protein (GFP) under the respective promoters.

**Materials and Methods**

*Animals*

All animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health. All animals were housed under standard laboratory conditions (12:12 hr light cycle, lights on at 0700; 22 ± 2°C). Food and water were available *ad libitum* except where noted. Male Sprague-Dawley rats weighing 300-350g were housed individually in hanging wire-mesh cages. All mice were group housed. Both male and female mice were used for experiments. GAD-GFP mice were obtained from The Jackson Laboratory (FVB-Tg(GadGFP)45704Swn/J). ObRb-GFP transgenic founder mice were obtained from Dr. Martin Myers, and offspring were produced by mating mice homozygous for Rosa26-GFP and LRb-Cre transgenes. POMC-GFP mice were obtained by mating POMC-Cre mice (gift from Dr. Bradford Lowell) (2) with Z/EG-EGFP reporter mice from Jackson Laboratory (22).
Peptides and antibodies

Cholecystokinin (sulfated octapeptide, CCK 26-33) was purchased from Bachem-Penninsula (Cat. # 7183; San Carlos, CA). MEK inhibitor U0126 was purchased from Promega (Madison, WI; Cat. # V1121). Recombinant mouse leptin (vial # AFP565) was obtained through NHRR, NIDDK. A complete list of primary antibodies is provided in Table 1.

Immunohistochemical detection of phospho-ERK 1/2 and double labeling protocols

Rats or mice, 4 animals per group, were adapted to handling and fasted overnight. On the test day, animals were injected with saline or CCK (5 or 10 μg/kg in 0.9% saline). Twelve minutes after the injection, animals were anesthetized with pentobarbital sodium (85 mg/kg, ip), and 3 min later transcardially perfused with heparinized (20U/ml) saline, followed by ice-cold 4% phosphate-buffered (pH 7.4) paraformaldehyde. The brains were extracted, blocked and post-fixed for a minimum of 2 hr. Tissue was then cryoprotected in 15% sucrose in 0.1M phosphate buffered saline (PBS) overnight at 4°C. Coronal sections of the medulla were cut on a cryostat at 20 μm for mouse and 30 μm for rat, separated into five series, and stored in a cryoprotectant solution (50% PBS, 30% ethylene glycol, 20% glycerol) at -20°C. One series from each animal, covering the rostro-caudal extent of the NTS, was processed for pERK1/2 immunohistochemistry. With appropriate washes between incubations, free-floating sections were rinsed with fresh 0.5% sodium borohydride in PBS followed by pretreatment in a blocking solution of 5% normal donkey serum in PBS with 0.5% Triton X-100. Rabbit anti phospho-p44/42 MAP Kinase in PBS/Triton X-100 was applied at a 1:1000 concentration overnight at room temperature. Sections were incubated in Cy3-conjugated donkey anti-rabbit antibody (1:500; Jackson ImmunoResearch, West Grove, PA). A second series of sections was processed for pERK1/2 immunoreactivity using nickel-enhanced diaminobenzidine as the chromogen as described previously (36). Nonspecific staining was absent when the primary antibody was omitted.

Sections of the rat brainstem processed for pERK1/2 and TH double labeling were incubated in rabbit anti-pERK antibody diluted 1:1000 and mouse anti-TH antibody diluted 1:500 overnight at room temperature. Sections were then incubated in Cy3-conjugated donkey anti-rabbit antibody (1:500) and Cy2-conjugated donkey anti-mouse antibody (1:500). For pERK1/2 and nNOS double labeling, rat
brainstem sections were incubated in rabbit anti-pERK antibody (1:1000) and mouse anti-nNOS antibody diluted 1:500 overnight at room temperature, followed by incubation in Cy3-conjugated donkey anti-rabbit and Cy2-conjugated donkey anti-mouse antibody (1:500).

Since POMC, ObRb and GAD-containing neurons cannot easily be demonstrated in the brainstem using conventional immunohistochemical techniques, transgenic mice expressing green fluorescent protein (GFP) under either POMC, GAD or ObRb promoters were used. Fluorescent (Cy3) pERK 1/2 immunohistochemistry was performed as described for rat tissue. In some cases, when the GFP-label was weak, we enhanced the signal by using GFP antibody. To this end, sections of the brainstem were incubated in goat anti GFP antibody diluted 1:500, followed by biotinylated donkey anti goat antibody diluted 1:500 for 90 min, and 1% Alexa Fluor 488 for 30 min.

In addition to using ObRb-GFP mouse model, the location of leptin-responsive neurons was also determined by using leptin-induced expression of pSTAT3. For pERK 1/2 and pSTAT3 double immunohistochemistry, ObRb-GFP mice received either two injections of saline, saline followed by CCK (5 μg/kg) or injection of leptin (3 mg/kg) followed by injection of saline. Leptin injections were made 90 min before perfusion, whereas CCK injections were made 15 min before perfusion. Twelve minutes after the last injection, mice were anesthetized with sodium pentobarbital (85 mg/kg,ip) and 3 min later transcardially perfused. Sections through the NTS were processed for pSTAT3 immunohistochemistry according to the protocol of Munzberg (21). Sections were incubated in 1% NaOH and 1%H₂O₂ in potassium PBS (KPBS) for 20 min, followed by 0.3% glycine for 10 min and in 0.03% SDS in KPBS for 10 min. Sections were incubated in 4% normal donkey serum in 0.4% TrixonX-100 in KPBS for 2 hours and placed in rabbit anti-pSTAT3 antibody diluted 1:1000. Tissue was washed in 1% normal donkey serum in 0.02% KPBS and placed in biotinylated donkey anti-rabbit antibody (1:250; Jackson ImmunoResearch) for 1 hour. Sections were rinsed and placed in avidin-biotin complex (ABC; Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). Reaction product was visualized by placing sections in nickel-enhanced diaminobenzidine (Pierce, Rockford, IL). Following pSTAT3 immunolabeling, sections were processed for pERK1/2 immunohistochemistry as described above using Cy3 as the chromagen.

Neurons immunoreactive for any of the antigens used were counted using conventional light or fluorescence microscopy with a 20X objective. The number of single- or double-labeled neurons was
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counted bilaterally on 2-3 sections at three rostro-caudal levels of the NTS, immediately rostral to, at the level of the area postrema (AP) and immediately caudal to the AP. The average count of labeled neurons at each rostrocaudal level was calculated for each experimental group. In addition, the average count of pERK1/2-labeled neurons per section for each subnucleus of the NTS was calculated. Since POMC-GFP neurons were observed only in the caudal aspect of the NTS, two series of 20 μm sections through the caudal NTS were processed for pERK 1/2 and POMC-GFP double labeling. Counts were analyzed by student’s t test. In cases where more than two groups were compared, counts were analyzed by an ANOVA followed by a student’s t test. Results are presented as means ± SEM. Differences between groups were considered statistically significant at p < 0.05. In addition, the location of immunoreactive neurons was mapped onto a standard set of drawings of the NTS. Nomenclature of Paxinos (25, 26) was used for rat and mouse.

Effects of MEK inhibitor on CCK-induced stimulation of pTH

Animals were anesthetized with ketamine/xylazine/acepromazine (80/5/1.6 mg/kg sc), and given atropine (1 mg/kg ip). A 24-Ga stainless steel guide cannula was aimed at the fourth ventricle (2.5 mm anterior to the posterior occipital suture, on the midline, 5.0 mm below the dura). A 30-Ga beveled injector was designed to protrude 1.0 mm from the guide cannula. Rats were given 10 days to recover, after which cannula placement and patency were verified using the 5-thio-glucose test (32), consisting of injecting 5-thio-glucose (210 μg in 3 μl sterile saline) and measuring plasma glucose concentration after 30 minutes. Only animals responding with an increase of plasma glucose concentration of at least 80 mg/ml were used for experiments.

Sixteen cannulated rats were used in a crossover, counterbalanced design to study the effect of the MEK inhibitor U0126 on CCK-induced stimulation of pTH. At 0800 on test days, overnight-fasted rats were given U0126 (2 μg in 50% DMSO/sterile saline) or vehicle, by infusing 3 μl into the 4th ventricle over a 2 min period. After infusion, the animals were returned to their home cages and one hour later given intraperitoneal injections of saline or CCK (2 μg /kg, ip). Fifteen min after the ip CCK or saline injection rats were killed to harvest NTS tissue.
Tissue dissection for protein purification

Rats were killed by guillotine and brains were extracted and snap frozen in isopentane cooled in dry ice. Brains were extracted and frozen approximately two minutes after decapitation. Frozen brains were mounted on chucks and slowly warmed to −20°C in a cryostat. Three hundred μm-thick sections were cut and the NTS was dissected on a cold plate by means of cylindrical stainless steel punch tools. Tissue from each rat was then solubilized in 2% SDS and total protein was determined by the bicinchoninic acid (BCA) assay. The solubilized protein was aliquotted and stored at −80°C. Punching an area of the NTS bordered by the solitary tracts, the area postrema, the dorsal motor nucleus, and the nucleus cuneatus and gracilis on six slices, spanning a rostrocaudal level from −13.0 mm to −14.8 mm from bregma yielded an average of 200 μg of total protein per rat.

Western blotting

Western blotting was performed as described previously. An aliquot of the frozen sample was diluted with an equal volume of 2X electrophoresis sample buffer and boiled for 10 min. Twenty micrograms was separated by size on a 10% SDS-polyacrylamide gel using the Laemmli buffer system and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes in Towbin-transfer buffer. After transfer, blots were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST). The membranes were blocked in PBST containing 5% non-fat dry milk and 1% bovine serum albumin for 1 hr at room temperature with agitation. Immunoblotting with phospho-specific antibodies was carried out after transfer of proteins to PVDF membranes. Membranes were incubated for 1 hr at room temperature with a specific phosphorylated rabbit polyclonal primary antibody (pERK 1/2; 1:1000), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (diluted to 1:1000, Southern Biotechnology, Birmingham, AL) for 1 hr at room temperature. After this incubation, the membranes were washed in PBST, the antigen-antibody-peroxidase complex detected by enhanced chemiluminescence, and visualized by exposure to Amersham-Pharmacia Biotech Hyperfilm ECL. Membranes were then stripped by incubation in stripping buffer for 30 min at 50°C with gentle agitation. Membranes were blocked and re-probed with total rabbit polyclonal antibodies to pTH(Ser-31). Film autoradiograms were analyzed and quantified by computer-assisted densitometry.
Data analysis

Western blots were quantified using densitometry (HP Scanjet 5200, and BioRad Quantity One software 4.4.1). Densitometry scores were analyzed by two-way ANOVA followed by Bonferroni-adjusted least-squared-difference post hoc comparisons (SYSTAT-10). Counts of pERK-positive neurons in the NTS were analyzed by Student’s t-test. Results are presented as means ± SEM.

Results

Intraperitoneal CCK administration induces phosphorylation of ERK 1/2 in the NTS of rats and mice

In both rats and mice, exogenous administration of CCK increased the number of pERK1/2 immunoreactive neurons in the NTS compared to control saline injection (Figs. 1-3). In saline-treated control rats and to a lesser extent in mice, a moderate number of neurons spontaneously expressed pERK, particularly in the medial, intermediate, and commissural subnuclei. Administration of either 5 or 10 μg/kg of CCK in rats significantly increased numbers of pERK-positive neurons in the medial and commissural subnuclei (Fig. 3). There were no differences in the number or in the distribution of pERK1/2 immunoreactive neurons between rats that received 5 or 10 μg/kg of CCK (Fig. 3). In the mouse, the number of CCK-induced pERK-positive neurons was generally lower compared to the rat. Administration of 5 μg/kg of CCK significantly increased the number of pERK1/2 immunoreactive neurons in the medial, gelatinous, and ventrolateral subnuclei (Figs. 2, 3).

Labeled cells were of variable size and the label typically extended to one or more distal dendrites (for higher magnification examples, see Figs. 4, 7, and 9). Thus, although we have not used cell type-specific markers for positive proof, we assume that most labeled cells are neurons.

CCK induces phosphorylation of ERK1/2 and tyrosine hydroxylase in a population of catecholaminergic NTS neurons in the rat

Some pERK 1/2 immunoreactive neurons in rats also expressed the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) (Figs. 4 and 5). Although some double-labeled neurons were also found after control saline injections, both the number (10.5 ± 0.8, vs. 16.0 ± 1.5 p<0.05) of double-labeled neurons and the percent of total TH-positive neurons (20.4 ± 2.4 vs. 29.4 ± 1.7 in the NTS, p< 0.05) was
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significantly higher after 10 μg/kg CCK (Fig. 5). However, double-labeled neurons represented only a fraction of about 20% of the total number of neurons expressing pERK following CCK administration.

Because TH has a selective ERK phosphorylation site (12), and given CCK’s ability to stimulate the ERK-pathway in TH expressing neurons, we speculated that CCK would also stimulate TH activity via the ERK-pathway. In rats, CCK (2 μg/kg, ip) produced a significant 3-fold increase (p< 0.05) in phosphorylated Ser(31)TH compared to control saline injection (Fig. 6). To test mediation of Ser(31)TH phosphorylation by the ERK-pathway, we pretreated some rats with 4th ventricular injections of the MEK inhibitor U0126 (2 μg/rat). Under these conditions, the CCK stimulatory effect was greatly attenuated and no longer significantly different from intraperitoneal saline injection (Fig. 6). Injection of the MEK-blocker alone did not significantly affect baseline p-Ser(31) TH.

**CCK induces pERK phosphorylation in a fraction of POMC, but not in GAD67 and nNOS-expressing neurons**

In an attempt to characterize the neurochemical phenotype of the many non-catecholaminergic NTS neurons exhibiting CCK-induced pERK, we used double-labeling strategies for the visualization of POMC, leptin receptors (ObRb), and the candidate neurotransmitters GABA and nNOS. Because of the inherent difficulty to immunohistochemically demonstrate POMC, GABA, and ObRb in the medulla, we used GFP-expressing mouse models.

POMC-GFP neurons were observed in the caudal portion of the mouse NTS, primarily in the commissural subnucleus (Fig. 7). Quantitative analysis showed that CCK administration significantly increased the number of POMC neurons expressing pERK (5.8 ± 2.3 vs. 14.6 ± 2.3 neurons/section, p<0.05) and the number of double-labeled neurons expressed as percent of POMC neurons (11.5 ± 4.3 vs. 34.5 ± 7.6 %, p<0.05) compared with saline (Fig. 8).

The GFP-labeled GAD67 mouse model was used to visualize GABA containing neurons. Although there were many GFP-labeled GAD67 neurons throughout the NTS, very few (< 1%) expressed pERK and this percentage was not different in saline and CCK-treated mice. (Fig. 9 a,b).

Neuronal NOS was stained immunohistochemically in CCK-injected rats, but again, pERK 1/2 immunoreactivity was colocalized in few neurons, mainly in the rostral NTS. CCK-induced pERK was
almost absent from the central subnucleus of the NTS, which contains a high density of NOS neurons (Fig. 9 c,d).

GFP-labeled ObRb immunoreactive neurons were observed in the NTS of mice, with the majority of these neurons localized at the level of the area postrema (Fig. 10, Fig. 11). However, only a few of these neurons also contained pERK1/2 immunoreactivity after CCK administration (Fig. 10). Because we found relatively few GFP-labeled ObRb neurons in the NTS, a group of mice also received intraperitoneal injections of leptin, and sections of the NTS were labeled for pSTAT3, a marker for leptin-responsive neurons (21). As described previously (9, 14, 15), leptin injections induced expression of pSTAT3 in the NTS. Although only about 25% of all pSTAT3 immunoreactive neurons also contained GFP, nearly all GFP neurons were positive for pSTAT3 (Fig. 10). However, only a small number of pERK 1/2 immunoreactive neurons also contained either pSTAT3 or GFP (Fig. 11). Injections of CCK alone had no effect on the number of GFP-labeled or pSTAT3 immunoreactive neurons and leptin treatment prior to CCK injections did not alter the number of pERK 1/2 immunoreactive neurons in the NTS (Fig. 11).

Discussion

Activation of pERK by CCK in the NTS has previously been implicated in the control of satiation (36). The aim of this study was to identify the phenotype of NTS neurons that show CCK-induced ERK activation. The results demonstrate that peripheral CCK administration increases the expression of pERK1/2 in a fraction of TH- and POMC-containing NTS neurons. In contrast, NTS neurons expressing nNOS and GAD67, as well as leptin-responsive and leptin receptor-expressing neurons did not show increased pERK1/2 activation following peripheral CCK administration. Together with our previous finding that 4th ventricular administration of an ERK-pathway blocker attenuated CCK-induced suppression of food intake (36), the results suggest that CCK-induced satiation is partly mediated through activation of the ERK-pathway in NTS neurons expressing TH and POMC, but not in neurons expressing nNOS, GAD67, or leptin receptor.

However, since only a small fraction of neurons exhibiting CCK-induced pERK also expressed TH or POMC, it is likely that other neurons of an unknown neurochemical phenotype are also involved. Identification of transmitters, peptides, receptors, and other functional markers in NTS neurons has been
a challenge. In studies demonstrating CCK’s neuronal activation pattern in the NTS with c-Fos, a similarly large fraction of activated neurons remained neurochemically undefined. However, TH- (28) and POMC- (10) expressing NTS neurons have been strongly implicated in the control of satiation, and the present results suggest that ERK-signaling in these neurons is involved.

Some differences in the location of NTS neurons activated by CCK were observed between rats and mice. In rats, CCK increased the number of pERK1/2 immunoreactive neurons only in the commissural and medial subnuclei of the NTS, whereas in mice, CCK increased the number of pERK1/2 immunoreactive neurons in the gelatinous, medial, and ventrolateral subnuclei of the NTS. While reasons for these differences are not readily apparent, previous reports have also shown species differences in the neurochemistry of NTS neurons (15). Distribution of GLP-1 immunoreactive neurons in the NTS has been shown to differ between rats and mice. Additionally, leptin injections induced expression of pSTAT3 in GLP-1 immunoreactive neurons in mice, but not in rats (15). Taken together, these observations suggest that differences in the distribution of pERK 1/2 immunoreactive neurons in the NTS may reflect either differences in the sensitivity of the two species to CCK or differences in anatomical distribution of neurons that respond to CCK. The possibility also exists that species differences observed in the present study were due to the strain of mouse used. Although direct comparison of the location of different neuronal populations between different strains of mice cannot be made, location and number of neurons in the NTS expressing pERK1/2 after injections of CCK did not differ between strains used in this study.

The major findings of the present study are that intraperitoneal injections of CCK increase the number of TH and POMC neurons expressing pERK 1/2 and induces an ERK-mediated phosphorylation of TH in the NTS. In contrast, CCK injections did not increase phosphorylation of ERK 1/2 in NTS neurons expressing nNOS, GAD67, or in leptin-responsive neurons. It has previously been shown that CCK increases the expression of c-Fos in TH immunoreactive neurons in the NTS (30, 31) and stimulates catecholamine release at their projection sites (8). It has also been demonstrated that TH-immunoreactive neurons partly mediate satiating effects of CCK (28). The present observations extend these previous findings by implicating participation of the ERK signaling pathway in a subpopulation of catecholaminergic NTS neurons. The results suggest that ip injected CCK leads to activation of the MEK → ERK signaling cascade and subsequent stimulation of tyrosine hydroxylase production. The facts that the ability of
exogenously administered CCK to suppress food intake depends on the integrity of abdominal vagal afferents (20, 34) and NTS catecholamine neurons (28), suggests that catecholamine neuron activation is mediated by vagal afferents, probably via the release of glutamate from central terminals of vagal afferents and NMDA and other glutamate receptors on catecholaminergic NTS neurons (1, 5). However, we cannot rule out direct action of circulating CCK on NTS neurons through both pre- and postsynaptic mechanisms in the present experiments. Such mechanisms are suggested from medulla slice preparations in rats and mice (1, 3) and from an inability of vagal deafferentation to completely inhibit the food intake suppressing effects of higher doses of CCK (20).

The roughly 20% of TH neurons displaying CCK-induced pERK1/2 immunoreactivity in our study is similar to the proportion of TH neurons that expressed c-Fos induced by gastric balloon distension (40) or ingestion of a satiating meal (29).

POMC-containing neurons in the NTS have been implicated in mediating satiating effects of CCK (10, 18). Peripheral CCK injections have been shown to induce c-Fos immunoreactivity in POMC neurons in the NTS and administration of the melanocortin receptor antagonist SHU9119 into the fourth ventricle attenuated CCK-induced inhibition of food intake (10). In addition, overexpression of the POMC gene in the NTS has been shown to reduce food intake, body weight, and visceral adiposity in a rat model of adult-onset obesity (18). Our finding of a small but significant number of POMC neurons exhibiting CCK-induced ERK-phosphorylation suggest that satiating effects of CCK may be partly mediated by ERK signaling in POMC neurons.

It has been shown previously that leptin and CCK have synergistic effects on food intake (4) and on the expression of c-Fos in the NTS (38), and we hypothesized that CCK would induce pERK 1/2 in leptin-responsive neurons in the NTS. However, pERK 1/2 was not induced in either ObRb-GFP-positive neurons or in neurons that expressed pSTAT3 after injections of leptin. These findings suggest that leptin and CCK activate separate populations of neurons in the NTS and that synergistic effects occur downstream of these neurons. Alternatively, synergistic effects may occur through another intracellular signaling cascade. In support of this suggestion, it has been demonstrated that both leptin and CCK can activate several signaling cascades including p38 and JNK (13, 39). Since leptin and CCK act synergistically to induce the expression of c-Fos in NTS neurons, it is likely that CCK, either through vagal
afferents or directly, activates these alternate signaling pathways in leptin-responsive neurons, leading to the induction of c-Fos.

Leptin-responsive neurons were identified by the use of GFP-reporter gene and by expression of pSTAT3 induced by leptin injections (9). Consistent with a previous report (17), the number of GFP-expressing neurons in the current study was only a fraction of neurons that expressed pSTAT3 following leptin injections. Although the reason for this difference between the numbers of pSTAT3 and GFP positive neurons is not clear, the expression levels of ObRb and consequently GFP in the brainstem may be very low. Alternatively, leptin may be inducing expression of pSTAT3 in some neurons indirectly, through release of another cytokine capable of activating the STAT3 pathway.

Although not every leptin-responsive neuron was labeled with GFP, every GFP-containing neuron also expressed pSTAT3 following leptin injections. This observation suggests that every neuron containing GFP responds to leptin and contains a functional leptin receptor.

Perspectives and Significance

A key role for the hindbrain in the control of food intake has long been recognized, but the specific neural pathways and signaling mechanisms remain ill defined. Clearly, changes in feedback from pre and postabsorptive mechano- and chemo-sensors to the caudal brainstem eventually lead to changes in oromotor and autonomic outflow either resulting in readiness to eat more or to stop eating. While considerable progress has been made identifying the sensory and motor limbs of these basic reflex arcs, much less is known about how sensory information is processed and leads to meaningful motor action. Specifically applied to satiety, we do not know how feedback from the gut is integrated with input from the gustatory system and information from higher brain areas, eventually leading to meal termination and a period of not eating. These integrative processes are likely performed by signaling steps both within specific NTS neurons, and between neurons constituting the extended circuitry. Our previous work has demonstrated that the cAMP → ERK → CREB signaling cascade in NTS neurons is involved in the integration of at least two important determinants of meal size, CCK and MC4 receptor melanocortin signaling (6). Here, we show that CCK-induced ERK signaling preferentially takes place in NTS neurons expressing certain transmitters and peptides. These findings corroborate other work implicating
catecholamine and POMC expressing NTS neurons in mediating food satiation. However, because only a relatively small portion of neurons exhibiting CCK-induced activation of the ERK pathway is of these two phenotypes, significant questions remain. What is the neurochemical phenotype of the majority of activated neurons, and is this phenotype important for satiety? Would a more natural and complete activation of feedback from the gut such as after a full meal activate a larger portion of catecholamine and POMC neurons? What is the role of other intracellular signaling pathways in satiety? Further identification of the rules and mechanisms of signal integration in the solitary nucleus will be important for understanding the process of food satiation, with implications for the development of appetite suppressant and anti-obesity drugs.
Table 1. Primary antibodies used

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Figure Legends

**Figure 1.** Photomicrographs showing the distribution of pERK immunoreactive neurons in the dorsal vagal complex of rat (a,b) and mouse (c,d) following intraperitoneal injection of CCK (a,c) or saline (b,d). Scale bar, 250 μm. For abbreviations, see Fig. 2.

**Figure 2.** Distribution of CCK-induced pERK immunoreactive neurons across three rostro-caudal levels of the dorsal vagal complex of the rat (left) and mouse (right). For clarity, each pERK immunoreactive neuron in a typical section from one animal is represented by a black dot. Distance from bregma is indicated for each section according to the rat atlas of Paxinos and Watson (26) and the mouse atlas of Paxinos and Franklin (25). Cen, central; Com, commissural; DM, dorsomedial; Gel, gelatinosus; IM, intermediate; Int, interstitial; Med, medial; VL, ventrolateral subnuclei of NTS; AP, area postrema; cc, central canal; Gr, gracilis; 4v, 4th ventricle; 10, dorsal motor nucleus; 12, hypoglossal nucleus.

**Figure 3.** Quantitative analysis of pERK immunoreactive neurons in rat and mouse NTS subnuclei following intraperitoneal injections of CCK or saline. Average number of neurons per section in each subnucleus was computed. * p < 0.05 compared to respective saline control (based on ANOVA followed by Bonferroni-adjusted multiple comparisons test). For abbreviations, see Fig. 2.

**Figure 4.** Colocalization of CCK-induced pERK in catecholaminergic neurons of the rat NTS. Lower (a-a’)- and higher (b-b’)-power confocal image stacks (15 x 1.5 μm) were taken separately for pERK immunoreactivity (red) and tyrosine hydroxylase (TH) immunoreactivity (green), and merged images are shown on the right. Scale bar in a: 50 μm for a-a”, 25 μm for b-b”.

**Figure 5.** Quantitative analysis of pERK expression in catecholaminergic neurons of the rat NTS following either saline (white bars) or CCK (10 μg/kg, ip; black bars). Average number per section of pERK and TH-immunoreactive neurons are shown on the left. Numbers of double-labeled neurons and numbers
expressed as percentage of TH and pERK neurons are shown on the right. * p < 0.05, based on individual t-tests.

**Figure 6.** CCK-induced phosphorylation of tyrosine hydroxylase is partially mediated through the ERK-pathway. Effect of saline or CCK (2 μg/kg, ip) on phosphorylation of tyrosine hydroxylase at Ser31 was measured in the absence or presence of 4th ventricular pretreatment with the MEK-inhibitor U0126 (10 μg) in rats. Representative immunoblots of micro-punched NTS tissue are shown on top. Means ± SEM of optical density of immunoblots is shown on bottom. Bars that do not share a common letter are statistically (p < 0.05) significantly different from each other (based on ANOVA followed by Bonferroni-adjusted multiple comparisons test).

**Figure 7.** Colocalization of pERK in NTS neurons expressing POMC in mice injected with CCK (10 μg/kg). Lower (a-a’’) and higher (b-b’’) power confocal images showing pERK and POMC-GFP separately and merged. Examples of double-labeled neurons are indicated by arrows. Confocal image stacks consisted of 15 x 1.5 μm for a, and 6 x 1 μm for b. The distribution of POMC-GFP neurons in the NTS is shown in c. Scale bar in c: 60 μm for a-a’’, 20 μm for b-b’’. For abbreviations, see Fig. 2.

**Figure 8.** Quantitative analysis of pERK expression in POMC-GFP neurons of the mouse caudal NTS following either saline (white bars) or CCK (10 μg/kg, ip; black bars) injections. Numbers of pERK and POMC-GFP immunoreactive neurons per section are shown on the left. Numbers of double-labeled neurons and percentage of POMC-GFP and pERK neurons are shown on the right. Neurons were counted in the caudal NTS from two series of sections. * p < 0.05, based on individual t-tests.

**Figure 9.** Absence of colocalization of pERK in GAD67- and nNOS-expressing NTS neurons in mice or rats administered CCK. Merged confocal image stacks (15 x 1.5 μm) showing pERK (red) and GAD67-GFP (a, b), or nNOS (c, d, green). Scale bar in d: 200 μm for a, b; 100 μm for c,d.
Figure 10. Absence of colocalization of pERK and leptin receptor in NTS neurons in mice administered CCK (5 µg/kg, ip). a,b: Merged confocal image stacks (15 x 1.5 µm) showing pERK (red) and ObRb-GFP (green). c: Merged confocal image stack (15 x 1.5 µm) showing pERK (red) with transmitted light image showing pSTAT3 (black cell nuclei on green background). Arrow indicates a rare double-labeled neuron. d: Merged image of confocal stack (15 x 1.5 µm) showing ObRb-GFP (green) with transmitted light image showing pSTAT3 (red cell nuclei). Scale bar in b: 50 µm for a and c; 25 µm for b and d.

Figure 11. Quantitative analysis of colocalization of CCK-induced pERK and leptin receptor in the NTS of ObRb-GFP mice. Leptin receptor was visualized either by transgenic expression of GFP (ObRb-GFP) or leptin-induced pSTAT3 immunohistochemistry. Average number of neurons per section was computed. Percent of total GFP-positive neurons and of total pSTAT3-positive neurons that were double labeled is shown on the right. * p< 0.05 compared to saline control based on individual t-tests.
References


Fig. 1

Rat

Mouse

10 μg/kg CCK

5 μg/kg CCK

Saline

Saline

com

ts

x

cc

bar
Fig. 2
Fig. 3
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.
Fig. 8.
Fig. 10.
Fig. 11.