

## Estradiol treatment increases CCK-induced c-Fos expression in the brains of ovariectomized rats

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**Eckel, Lisa A., Thomas A. Houppt, and Nori Geary.** Estradiol treatment increases CCK-induced c-Fos expression in the brains of ovariectomized rats. *Am J Physiol Regul Integr Comp Physiol* 283: R1378–R1385, 2002; 10.1152/ajpregu.00300.2002.—The ovarian hormone estradiol reduces meal size and food intake in female rats, at least in part by increasing the satiating potency of CCK. Here we used c-Fos immunohistochemistry to determine whether estradiol increases CCK-induced neuronal activation in several brain regions implicated in the control of feeding. Because the adiposity signals leptin and insulin appear to control feeding in part by increasing the satiating potency of CCK, we also examined whether increased adiposity after ovariectomy influences estradiol's effects on CCK-induced c-Fos expression. Ovariectomized rats were injected subcutaneously with 10  $\mu\text{g}$  17 $\beta$ -estradiol benzoate (estradiol) or vehicle once each on Monday and Tuesday for 1 wk (*experiment 1*) or for 5 wk (*experiment 2*). Two days after the final injection of estradiol or vehicle, rats were injected intraperitoneally with 4  $\mu\text{g}/\text{kg}$  CCK in 1 ml/kg 0.9 M NaCl or with vehicle alone. Rats were perfused 60 min later, and brain tissue was collected and processed for c-Fos immunoreactivity. CCK induced c-Fos expression in the nucleus of the solitary tract (NTS), area postrema (AP), paraventricular nucleus of the hypothalamus (PVN), and central nucleus of the amygdala (CeA) in vehicle- and estradiol-treated ovariectomized rats. Estradiol treatment further increased this response in the caudal, subpostremal, and intermediate NTS, the PVN, and the CeA, but not in the rostral NTS or AP. This action of estradiol was very similar in rats tested before (*experiment 1*) and after (*experiment 2*) significant body weight gain, suggesting that adiposity does not modulate CCK-induced c-Fos expression or interact with estradiol's ability to modulate CCK-induced c-Fos expression. These findings suggest that estradiol inhibits meal size and food intake by increasing the central processing of the vagal CCK satiation signal.

ingestive behavior; feeding; satiation; nucleus of the solitary tract; area postrema; paraventricular nucleus of the hypothalamus; amygdala; female rats

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OVARIAN ESTRADIOL EXERTS POTENT inhibitory effects on feeding in a variety of species. This action of estradiol is prominent in rats, in which spontaneous feeding is decreased during the nocturnal phase of estrus after the cyclic increase in estradiol secretion that begins in diestrus and peaks in the diurnal phase of proestrus (7,

17, 20, 37). This inhibition of feeding is mediated by a reduction in meal size, with meal frequency often increased (7, 17, 20, 37). Studies of ovariectomized rats directly link the estral reduction in meal size with estradiol. Ovariectomy tonically increases meal size, abolishes the cyclic estral reduction in nocturnal meal size, increases daily food intake, and contributes (together with decreases in locomotor and metabolic activity) to body weight gain (1, 7, 26, 27, 57). A cyclic regimen of estradiol treatment, designed to mimic the changes in plasma estradiol levels across the estrous cycle, normalized meal size, food intake, and body weight gain to the levels observed in gonadally intact rats (5). Thus estradiol apparently participates in the normal control of meal size in female rats.

Estradiol reduces meal size, at least in part, by increasing the potency of peripheral satiation signals involved in meal termination (26). The most extensively studied such interaction involves CCK, a peptide that is released from the small intestine during meals and binds to low-affinity CCK-1 receptors on vagal afferents of the pylorus and proximal duodenum to initiate a negative-feedback satiation signal (30, 54, 56). Estradiol treatment increases the satiating potency of exogenous CCK in ovariectomized rats (9, 26, 29, 39). More importantly, the satiating potency of endogenous CCK is increased during estrus in gonadally intact rats and by estradiol treatment in ovariectomized rats (3, 18).

Neurons that may participate in the central processing of meal-generated signals controlling food intake have been identified in several brain areas by immunohistochemical detection of c-Fos protein, the product of the immediate-early gene *c-fos* (50, 51). In male rats, intraperitoneal injection of CCK increases c-Fos expression in several brain regions including the nucleus of the solitary tract (NTS), area postrema (AP), dorsal motor nucleus of the vagus (DMNV), lateral parabrachial nucleus (LPBN), paraventricular nucleus of the hypothalamus (PVN), and central nucleus of the amygdala (CeA) (12, 38, 46, 51, 52). c-Fos immunohistochemistry has also been applied to the analysis of estradiol's inhibitory effects on feeding. We recently

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demonstrated that estradiol treatment increases feeding-induced c-Fos expression in the NTS, PVN, and CeA in ovariectomized rats (19). As reviewed above, CCK is an important component of the satiating action of ingested food, and estradiol increases the satiating potency of CCK. Therefore, in the present experiments, we used c-Fos immunohistochemistry to determine whether estradiol increases CCK-induced neuronal activation in ovariectomized rats.

We used a cyclic regimen of estradiol treatment introduced by McEwen and colleagues (43, 53). In this regimen, ovariectomized rats receive estradiol each Monday and Tuesday and are tested on Thursdays, which models estrus in intact rats. When progesterone is injected 4 h before tests, this regimen increases sexual receptivity (lordosis) to estrous levels (43, 53). In addition, even in the absence of progesterone, this estradiol regimen induces tonic and cyclic decreases in food intake and meal size (27, 44), increases running wheel activity (44), normalizes body weight (3, 27, 29, 44), and cyclically increases CCK's satiating potency (3, 29). Both the increased sexual receptivity and the decreased feeding are evident after the first pair of estradiol injections and recur similarly through months of repeated treatments (27, 44, 53).

In male rats, treatment with leptin or insulin increases exogenous CCK's satiating potency (6, 23, 41, 42, 49), and leptin increases CCK-induced c-Fos in the NTS, AP, and PVN (23). Leptin also increases c-Fos expression in the NTS and PVN after intragastric or intraduodenal nutrient infusions (22). These interactive effects of leptin are similar to the effects of estradiol on CCK satiation and food-induced c-Fos expression in ovariectomized rats. As leptin and insulin are thought to mediate the inhibitory effect of increased adiposity on feeding (25, 58), it is possible that increased adiposity alone might increase CCK-induced c-Fos expression in ovariectomized rats and obscure the effects of estradiol. Therefore, here we probed estradiol's effects on CCK-induced c-Fos expression both 1 and 5 wk postovariectomy, i.e., before and after ovariectomy-induced body weight gain.

## METHODS

### *Animals and Surgery*

Forty-one adult female Long-Evans hooded rats (Charles River Breeding Laboratories, Wilmington, MA; weighing 200–225 g at study onset) were housed individually in hanging cages with wire mesh floors. Rats were given free access to rat chow (Purina 5001, St. Louis, MO) and tap water unless otherwise noted. The room was maintained at  $20 \pm 2^\circ\text{C}$  with a 12:12-h light/dark cycle (dark onset 1400). Two red 25-W incandescent bulbs provided dim illumination during the dark period. After 1 wk of adaptation to these conditions, rats were anesthetized by intraperitoneal injection of 1 ml/kg of a mixture of 70 mg/ml ketamine (Ketaset, Fort Dodge, IA) and 4.5 mg/ml xylazine (Rompun, Mobay, Shawnee, KS) and bilaterally ovariectomized using an intra-abdominal approach. Estradiol treatment began 1 wk postoperatively. All procedures were approved by the Weill Medical College of Cornell University Institutional Animal Care and

Use Committee and comply with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings" (2).

### *Estradiol and CCK Treatment*

*Experiment 1: CCK-induced c-Fos expression in ovariectomized rats after acute estradiol treatment.* Ovariectomized rats were assigned to one of two groups of approximately equal body weight. One group ( $n = 10$ ) received intrascapular subcutaneous injections of  $10 \mu\text{g}$   $17\beta$ -estradiol-3-benzoate (estradiol; Sigma) in  $100 \mu\text{l}$  sesame oil vehicle (Sigma), and the other group ( $n = 9$ ) received  $100 \mu\text{l}$  vehicle alone. Injections were done on Monday and Tuesday at 1000. On Thursday, 22-h food-deprived rats were intraperitoneally injected with  $4 \mu\text{g}/\text{kg}$  CCK-8 (generously donated by the Bristol-Myers-Squibb Pharmaceutical Research Institute) or 1 ml/kg 0.15 M saline vehicle, 1 h after dark onset (1400). Rats were anesthetized 60 min after injection of CCK or saline, and brain tissue was collected and processed for c-Fos-like immunoreactivity as described below. At this time, estradiol-treated rats had gained  $13 \pm 4$  g since ovariectomized and vehicle-treated rats had gained  $21 \pm 3$  g, a nonsignificant difference,  $t(17) = 1.41$ ,  $P > 0.05$ .

*Experiment 2: CCK-induced c-Fos expression in ovariectomized rats after chronic estradiol treatment.* Ovariectomized rats were assigned to one of two groups of approximately equal body weight. One group ( $n = 12$ ) received intrascapular subcutaneous injections of  $10 \mu\text{g}$  estradiol in  $100 \mu\text{l}$  sesame oil vehicle each Monday and Tuesday for 5 wk, and the other group ( $n = 10$ ) received  $100 \mu\text{l}$  vehicle alone. On Thursday of the 5th wk of treatment, 22-h food-deprived rats were intraperitoneally injected with  $4 \mu\text{g}/\text{kg}$  CCK or 1 ml/kg 0.15 M saline vehicle and anesthetized and perfused as above. At this time, oil treated-rats had gained significantly more body weight than estradiol-treated rats,  $85 \pm 5$  vs.  $25 \pm 4$  g,  $t(20) = 9.15$ ,  $P < 0.0001$ .

### *Perfusion and Tissue Collection*

One hour after injection of CCK or saline, rats were anesthetized with pentobarbital sodium (65 mg/kg ip; Nembutal, Butler, Columbus, OH) and transcardially perfused with 100 ml of a heparinized 0.9% NaCl solution containing 0.5%  $\text{NaNO}_2$ , followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were then removed, post-fixed in paraformaldehyde for 2 h at room temperature, and cryoprotected in 30% sucrose solution at  $4^\circ\text{C}$  for 48 h. Hind-brain blocks were mounted on a freezing, sliding microtome, and 60 consecutive  $40 \mu\text{m}$  coronal sections were cut beginning at the obex and moving anterior. This area corresponds to the region between 14.4 and 12.0 mm posterior to bregma in the atlas of Paxinos and Watson (47). Forebrain blocks were cut similarly into 100 consecutive sections from the optic chiasm (0.1 mm posterior to bregma) through the median eminence (4.1 mm posterior to bregma).

### *Immunohistochemistry*

Every second hindbrain section and every fourth forebrain section were processed for c-Fos-like immunoreactivity. Free-floating tissue sections were washed in PBS, blocked in a PBS solution containing 0.2% Triton X-100 and 1% BSA for 30 min, washed in a PBS solution containing 0.5% BSA (PBS/BSA), and incubated 20 h at room temperature with rabbit polyclonal anti-c-Fos peptide antisera (Ab-5, Oncogene Sciences, Cambridge, MA) diluted 1:20,000 in PBS/BSA. Sections were then washed in PBS/BSA and incubated 1 h at

room temperature with a biotinylated anti-rabbit goat antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS/BSA. Bound secondary antibody was amplified during a 1 h incubation of the sections in an avidin-biotin complex (Vectastain ABC Elite Kit, Vector Laboratories) diluted 1:50 in PBS/BSA. Antibody complexes were visualized by immersing the tissue in 2% diaminobenzidine solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 5 min. This reaction was stopped by rinsing the sections in PB. Sections were then mounted on microscope slides and placed under coverslips with Permount (Fisher Scientific, Atlanta, GA).

#### Quantification of c-Fos-Like Immunoreactivity

The presence of c-Fos-like immunoreactivity was quantified using Image-Pro Plus software (V3.0, MediaCybernetics, Gaithersburg, MD). A constant set of threshold criteria based on optical density, object shape, and object size was used to identify c-Fos-positive cells containing dark, punctate, nuclear staining. c-Fos-like immunoreactivity was examined in several brain regions implicated in mediating the effects of estradiol, CCK and leptin, i.e., the NTS, the AP, the PVN, the CeA, the ventromedial hypothalamus (VMH), the medial preoptic area (MPOA), and the arcuate nucleus (ARC). To provide a more detailed account within the NTS, the first central relay of vagal afferent fibers, c-Fos expression was examined in the following four regions: caudal NTS (cNTS), including all sections caudal to the AP; subpostremal NTS (spNTS), including all sections in which the AP was visible; intermediate NTS (iNTS), including sections rostral to the AP in which the NTS abutted the fourth ventricle; and rostral NTS (rNTS), including sections in which the NTS diverged from the fourth ventricle. Every other hemisection through each of these regions was quantified. Templates delineating the mediolateral and dorsoventral borders of the brain regions of interest were based on Paxinos and Watson's (47) atlas. Only c-Fos-positive cells within these borders were counted.

#### Data Analysis

Data are presented as means  $\pm$  SE. In *experiment 1* (acute treatment), the numbers of c-Fos-positive cells were analyzed using separate two-factor ANOVAs (steroid treatment by peptide treatment) in each brain region. In *experiment 2* (chronic treatment), c-Fos data were first analyzed using separate three-factor ANOVAs (body weight gain by steroid treatment by peptide treatment) in each brain region because we were interested in the possibility that ovariectomy-in-

duced body weight gain might modulate CCK-induced c-Fos expression or estradiol's ability to increase such expression. These analyses failed to reveal any main or interactive effects of body weight gain, so the numbers of c-Fos-positive cells in *experiment 2* were reanalyzed using two-factor ANOVAs (steroid treatment by peptide treatment), as in *experiment 1*. When significant effects were detected by ANOVA ( $P < 0.05$ ), differences between individual means were examined with Tukey's honestly significant difference test. The standard error of the difference (SED) is reported as an estimate of experiment-wide residual variability. Data were analyzed with the BMDP (SOLO V. 6.0; SPSS; Chicago, IL) statistical package.

## RESULTS

### *Experiment 1: CCK-Induced c-Fos Expression in Ovariectomized Rats After Acute Estradiol Treatment*

There was little c-Fos expression in the NTS and AP after saline injection in both estradiol- and vehicle-treated rats. Intraperitoneal injection of CCK induced large increases in c-Fos expression in the cNTS, spNTS, and iNTS and a smaller increase in the rNTS (Figs. 1 and 2). In the cNTS, spNTS, and iNTS, estradiol treatment increased the number of CCK-induced c-Fos-positive cells but did not affect c-Fos expression in saline-treated control rats (Fig. 2, A-C); interaction effects,  $F(1,15) = 6.51-9.40$ ,  $P < 0.05$ , SED = 4-5 cells. The magnitude of the increase in c-Fos expression in estradiol-treated rats ranged from ~105 to 185%. In contrast, estradiol treatment did not affect CCK-induced c-Fos expression in the rNTS or the AP (Figs. 2D and 4A); main effects of CCK,  $F(1,15) = 4.71$ ,  $P < 0.05$ , SED = 1 cell, and  $F(1,15) = 7.39$ ,  $P < 0.01$ , SED = 11 cells, respectively; main effects of estradiol and interaction effects, not significant.

There were low levels of c-Fos expression in the PVN and CeA after saline injection in both estradiol-treated and untreated rats. Intraperitoneal injection of CCK increased c-Fos expression in the PVN and CeA (Figs. 3 and 4). In both regions, estradiol treatment increased the number of CCK-induced c-Fos-positive cells but did not affect c-Fos expression in saline-treated control rats (Fig. 4, B and C); interaction effects,  $F(1,15) =$

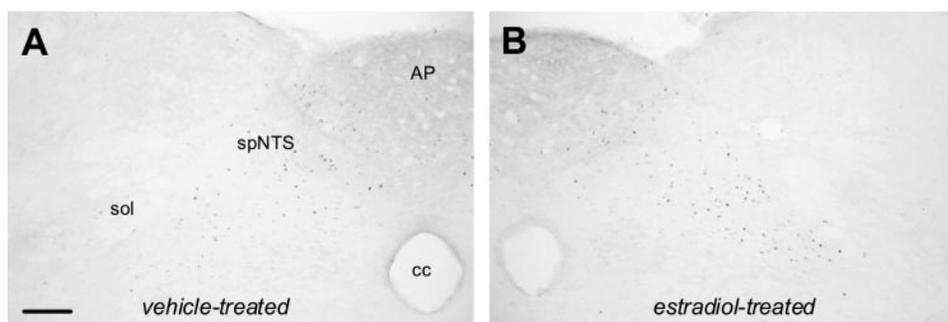


Fig. 1. Representative photomicrographs of coronal hemisections through the subpostremal nucleus of the solitary tract (spNTS) after intraperitoneal injection of 4  $\mu$ g/kg CCK in vehicle- and estradiol-treated ovariectomized rats in *experiment 1*. Rats were tested 2 wk postovariectomy, 2 days after subcutaneous injections of 10  $\mu$ g estradiol benzoate or sesame oil vehicle on 2 consecutive days. CCK induced large increases in c-Fos expression in the spNTS and area postrema (AP) of vehicle-treated (A) and estradiol-treated (B) rats. Estradiol treatment increased this response in the spNTS, but not in the AP. Scale bar = 100  $\mu$ m. sol, Solitary tract; cc, central canal.

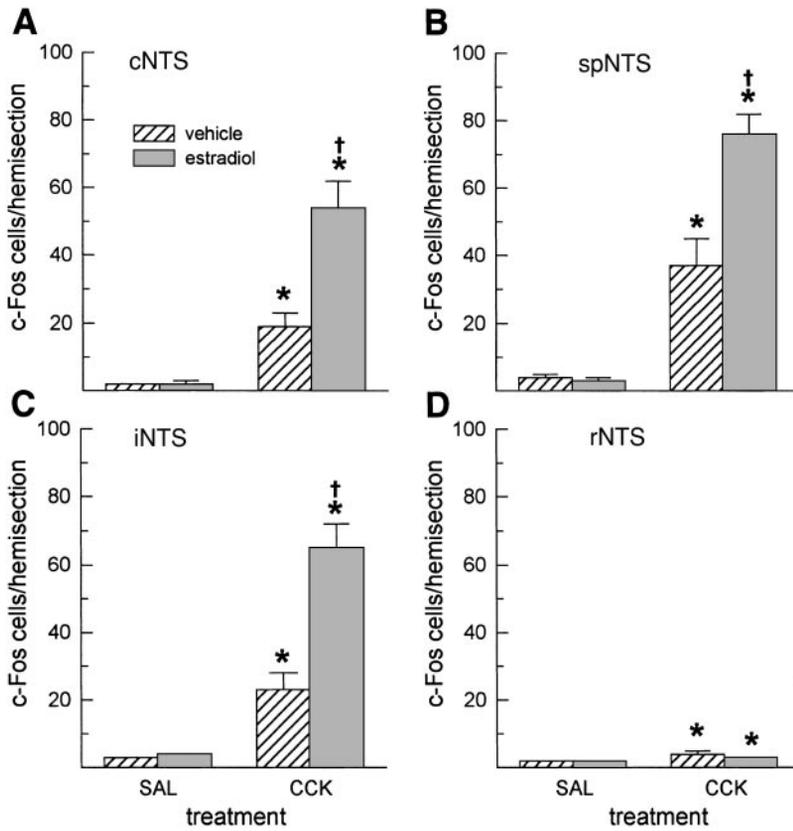


Fig. 2. Estradiol treatment increased CCK-induced c-Fos expression within regions of the NTS of ovariectomized rats. Data, means  $\pm$  SE, are from *experiment 1*, in which rats were tested 2 wk postovariectomy, 2 days after subcutaneous injections of 10 µg estradiol benzoate or sesame oil vehicle on 2 consecutive days. Intraperitoneal injection of 4 µg/kg CCK significantly increased c-Fos expression within each region of the NTS, relative to saline-treated (SAL) rats. Estradiol treatment significantly increased the amount of CCK-induced c-Fos expression in the caudal NTS (cNTS, A), subpostremal NTS (spNTS, B), and intermediate NTS (iNTS, C), but not in the rostral NTS (rNTS, D). \*Greater than saline-treated rats,  $P < 0.05$ ; †estradiol-treated rats greater than vehicle-treated rats,  $P < 0.05$ .

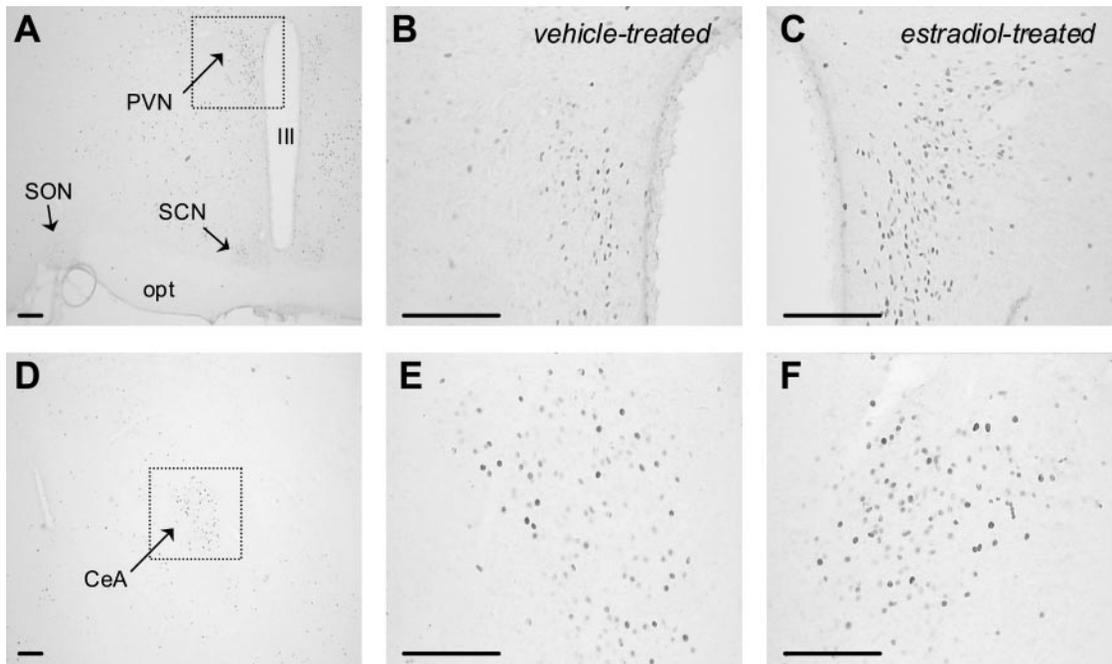


Fig. 3. Representative photomicrographs of coronal hemisections through the paraventricular nucleus (PVN; A-C) and central nucleus of the amygdala (CeA, D-F) after intraperitoneal injection of 4 µg/kg CCK in estradiol- and vehicle-treated rats in *experiment 1*, in which rats were tested 2 wk postovariectomy, 2 days after subcutaneous injections of 10 µg estradiol benzoate or sesame oil vehicle on 2 consecutive days. CCK induced large increases in c-Fos expression in the PVN and CeA. Estradiol treatment increased this response in both regions. Boxes in A and D depict the area of the PVN and the CeA photographed at higher magnification in (B, C, E, F). Scale bar = 100 µm. SON, supraoptic nucleus of the hypothalamus; SCN, suprachiasmatic nucleus; opt, optic tract; III, third ventricle.

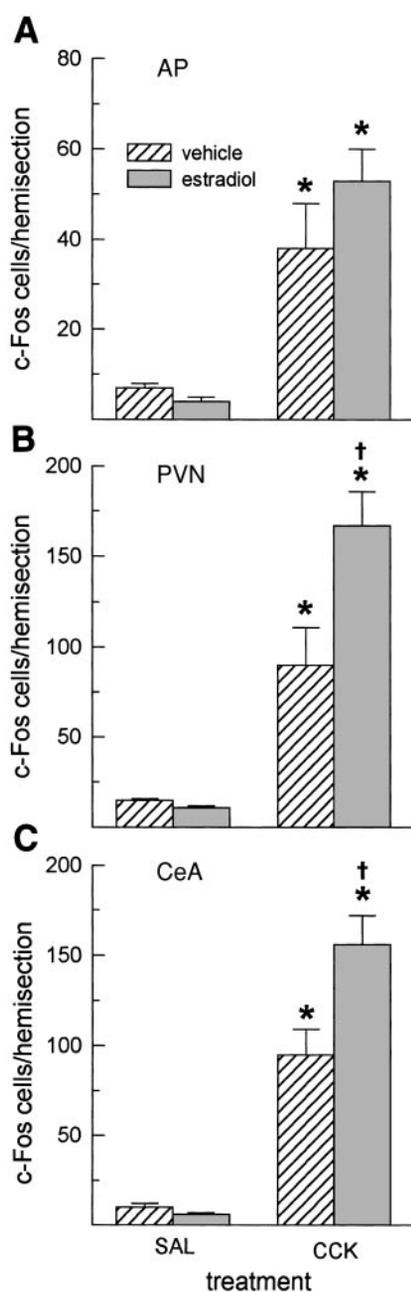


Fig. 4. Estradiol treatment increased CCK-induced c-Fos expression in the PVN and CeA, but not in the AP. Data, means  $\pm$  SE, are from *experiment 1*, in which rats were tested 2 wk postovariectomy, 2 days after subcutaneous injections of 10  $\mu$ g estradiol benzoate or sesame oil vehicle on 2 consecutive days. Intraperitoneal injection of 4  $\mu$ g/kg CCK increased c-Fos expression in the AP (A), PVN (B), and CeA (C) relative to saline-treated rats. Estradiol treatment significantly increased this response in the PVN and the CeA. \*Greater than saline-treated rats,  $P < 0.01$ ; †estradiol-treated rats greater than vehicle-treated rats,  $P < 0.05$ .

14.55 and 5.90,  $P < 0.05$ , SED = 15 and 11 cells, respectively. The magnitude of the increase in c-Fos expression in estradiol-treated rats was  $\sim 75\%$  in the PVN and  $\sim 50\%$  in the CeA. There were no apparent differences in the number of c-Fos-positive cells in the magnocellular or parvocellular subdivisions of the PVN. Examination of the VMH, MPOA, and ARC failed to reveal any c-Fos expression after CCK treatment.

### Experiment 2: Effects of Estradiol on CCK-Induced c-Fos Expression After Chronic Estradiol Treatment

The results closely paralleled those of *experiment 1*. Intraperitoneal injection of CCK induced large increases in c-Fos expression in the cNTS, spNTS, and iNTS, and a small increase in the rNTS (Table 1). In the cNTS, spNTS, and iNTS, estradiol treatment increased the number of CCK-induced c-Fos-positive cells but did not affect c-Fos expression in saline-treated control rats (Table 1); interaction effects,  $F(1,19) = 4.48$ – $5.32$ ,  $P < 0.05$ , SED = 5–7 cells. In contrast, estradiol treatment did not affect CCK-induced c-Fos expression in the rNTS or the AP (Table 1); main effects of CCK,  $F(1,19) = 10.46$ ,  $P < 0.01$ , SED = 1 cell, and  $F(1,19) = 23.71$ ,  $P < 0.001$ , SED = 6 cells, respectively; main effects of estradiol and interaction effects, not significant.

Intraperitoneal injection of CCK also increased c-Fos expression in the PVN and CeA (Table 1). In both regions, estradiol treatment increased the number of CCK-induced c-Fos-positive cells but did not affect c-Fos expression in saline-treated control rats (Table 1); interaction effects  $F(1,19) = 4.29$  and  $4.28$ ,  $P < 0.05$ , SED = 13 and 10 cells, respectively. There were no apparent differences in the number of c-Fos-positive cells in the magnocellular or parvocellular subdivisions of the PVN. Examination of the VMH, MPOA, and ARC failed to reveal any increase in c-Fos expression after CCK treatment.

### DISCUSSION

The satiating action of CCK on meal size is increased by estradiol treatment in ovariectomized rats (9, 26, 29, 39) and during estrus in intact, cycling rats (3, 18).

Table 1. CCK-Induced c-fos expression in the NTS, AP, PVN, and CeA of ovariectomized rats after chronic estradiol or vehicle treatment

	Treatment			
	Veh/Sal	E <sub>2</sub> /Sal	Veh/CCK	E <sub>2</sub> /CCK
Caudal NTS	5 $\pm$ 1	6 $\pm$ 1	21 $\pm$ 5*	68 $\pm$ 13*†
Subpostremal NTS	4 $\pm$ 1	4 $\pm$ 0	33 $\pm$ 8*	71 $\pm$ 8*†
Intermediate NTS	4 $\pm$ 1	4 $\pm$ 1	29 $\pm$ 6*	70 $\pm$ 11*†
Rostral NTS	1 $\pm$ 0	2 $\pm$ 1	3 $\pm$ 1*	4 $\pm$ 1*
AP	7 $\pm$ 1	4 $\pm$ 1	38 $\pm$ 10*	53 $\pm$ 7*
PVN	10 $\pm$ 2	6 $\pm$ 1	95 $\pm$ 14*	156 $\pm$ 16*†
CeA	15 $\pm$ 1	11 $\pm$ 1	90 $\pm$ 21*	167 $\pm$ 19*†

Data, means  $\pm$  SE, are from *experiment 2*. Ovariectomized rats received 5 wk of cyclic estradiol (2 daily subcutaneous injections of 10  $\mu$ g estradiol benzoate per week) or sesame oil vehicle treatment. Ovariectomy-induced body weight gain was significantly reduced by estradiol treatment. Two days after the final injections of estradiol or vehicle, rats were injected with CCK or saline and perfused 1 h later. Brain tissue was processed for c-Fos immunohistochemistry and the number of c-Fos-positive cells per hemisection was quantified. CCK increased c-Fos expression in the nucleus of the solitary tract (NTS), area postrema (AP), paraventricular nucleus (PVN), and central nucleus of the amygdala (CeA). Chronic estradiol treatment increased this response in the caudal, subpostremal, and intermediate NTS, the PVN, and the CeA. \* $P < 0.01$  compared with vehicle (Veh)/saline (Sal) and E<sub>2</sub>/Sal. † $P < 0.01$  compared with Veh/CCK.

Here, we investigated whether estradiol treatment would also increase CCK-induced c-Fos expression in the brain. We concentrated our search on the NTS, the AP, the PVN, the VMH, the MPOA, and the CeA, areas that have been implicated in the control of feeding by CCK or estradiol (10, 11, 15, 34, 51). In addition, because the putative adiposity signals leptin and insulin increase CCK's satiating potency (6, 23, 42, 49) and because leptin increases CCK's potency to stimulate c-Fos expression in the NTS and the PVN (23), we tested ovariectomized rats both before and after significant body weight gain. We also examined c-Fos expression in the ARC, the site of leptin receptors affecting feeding (23).

Our major finding was that estradiol treatment increased CCK-induced c-Fos expression in the cNTS, the spNTS, the iNTS, the PVN, and the CeA and that postovariectomy body weight gain did not appear to affect this action of estradiol. This parallels previous observations that estradiol increases the satiating potency of CCK in tests of scheduled and spontaneous feeding (9, 26, 29, 39). As increases in c-Fos expression reflect increases in neuronal activity, these data suggest that estradiol decreases meal size and food intake in part by increasing the central processing of the CCK satiation signal. We also found that CCK increased c-Fos expression in the rostral NTS and the AP, but these responses were not modulated by estradiol treatment. Finally, CCK failed to increase c-Fos expression in the VMH, MPOA, or ARC of either estradiol- or vehicle-treated rats.

Intraperitoneal injection of 4  $\mu\text{g}/\text{kg}$  CCK, which inhibits meal size by ~30–45% in female rats (24, 29), increased c-Fos expression in the NTS, AP, PVN, and CeA of ovariectomized rats, but in none of the other brain areas we investigated. This is similar to effects of 5–8  $\mu\text{g}/\text{kg}$  CCK on c-Fos expression in male rats (38). Larger (10–100  $\mu\text{g}/\text{kg}$ ) doses of CCK induce more intense c-Fos expression in these areas in male rats and also recruit c-Fos expression in additional areas, including the dorsal motor nucleus of the vagus, ventrolateral medulla, and supraoptic nucleus of the hypothalamus (40, 51). This is likely due to the nonsatiating, aversive effects of such doses of CCK (56).

Our findings extend previous analyses of CCK's effects on c-Fos responsiveness in male rats (12, 38, 46, 51, 52) to female rats and provide a more detailed account of the distribution of c-Fos expression induced by satiating doses of CCK within the NTS than did previous studies. The intense c-Fos expression in the cNTS, spNTS, and iNTS and relatively weak expression in the rNTS observed here is consistent with the projection of abdominal vagal afferents thought to mediate satiation. That is, the terminal fields of the majority of vagal afferent fibers from the stomach, small intestine, and liver are in the cNTS, spNTS, and iNTS, whereas oropharyngeal vagal afferents terminate in the rNTS (8, 45). This suggests that the effects of CCK on neuronal activation within the NTS observed here mimic the effects of feeding-related signals in the upper abdomen, including food stimuli causing CCK secretion,

increasing CCK-related vagal afferent electrophysiological activity, and causing satiation (54).

Estradiol treatment increased c-Fos expression elicited by CCK in the cNTS, spNTS, iNTS, PVN, and CeA. Because estradiol increases the satiating potency of CCK (9, 26, 29, 39), these increases in CCK-induced c-Fos in estradiol-treated rats may reflect increases in the activity of the neural networks mediating CCK satiation. The effects of food ingestion are parallel: CCK is an important mediator of the satiating action of ingested food (56), estradiol selectively increased the satiating action of intralipid, whose satiating effect is mediated by endogenous CCK (4), and estradiol increased the c-Fos expression that is induced by sweet milk ingestion in these same areas (19).

Our data extend two previous tests of the effects of estradiol on CCK-induced c-Fos expression. Flanagan-Cato et al. (24) reported that estradiol decreased CCK-induced c-Fos expression in the NTS and PVN of ovariectomized rats. It is difficult to suggest an explanation for the apparent discrepancy between these results and ours because the procedures of the two experiments were very similar. Our findings are consistent, however, with the report that estradiol treatment increased CCK-induced c-Fos expression in the NTS of ovariectomized mice (28). In this study, mice with an ER- $\alpha$  null mutation and their wild-type littermates were ovariectomized and implanted with slow-release pellets containing estradiol or vehicle. Intraperitoneal CCK injection increased c-Fos expression in the NTS of both ER- $\alpha$  knockout and wild-type mice, but estradiol treatment increased CCK-induced c-Fos only in the wild-type mice and not in the ER- $\alpha$  null mice, indicating that ER- $\alpha$  is necessary for this action of estradiol and that ER- $\beta$  alone is not sufficient (28).

ER- $\alpha$  is expressed in numerous areas of the rat brain (55). Because the NTS, PVN, and CeA are among these areas, our results are consistent with the possibility that estradiol may act in any of these sites to initiate its effect on meal size. The site(s) of the receptors mediating the estrogenic inhibition of feeding remains unclear. Some data implicate PVN estradiol receptors (10, 11), but other data (14, 36) are inconsistent with the hypothesis that PVN estradiol receptors are sufficient to account for the estrogenic inhibition of feeding.

CCK injection slightly increased c-Fos expression in the rNTS, but estradiol did not affect this response. This selectivity of estradiol to the cNTS, spNTS, and iNTS is consistent with other evidence that estradiol selectively increases the satiating potency of gastrointestinal food stimuli without affecting the control of meal size by oropharyngeal food stimuli (35). CCK injection markedly increased c-Fos expression in the AP, as has previously been reported (38, 40). This is similar to the effects of consumption of several foods (chow, balanced liquid diets, and sucrose solutions) on c-Fos expression in male rats (16, 50), but differs from the effect of sweetened condensed milk ingestion, which did not increase c-Fos expression in the AP in female rats (19). Estradiol tended to increase c-Fos expression in response to CCK, but this was not significant. Thus the

role of the AP in satiation and in estradiol's influence on satiation requires further study.

Leptin, the protein product of the *ob* gene, and pancreatic insulin are secreted in proportion to body adiposity and appear to function as feedback signals involved in the control of food intake and body adiposity (58). Like estradiol, both leptin and insulin reduce food intake in rats by selectively reducing meal size with little effect on meal frequency (21, 58). Again like estradiol, leptin and insulin appear to reduce meal size by altering the potency of meal-generated signals involved in the production of satiation (6, 23, 42, 49). Moreover, exogenous leptin administration in male rats increases the potency by which CCK induces c-Fos expression (23). This suggested to us that estradiol's effects on CCK-induced c-Fos expression might be different in less adipose ovariectomized rats, which presumably secrete less leptin and insulin, than in more adipose rats. But we saw no difference in the patterns of c-Fos expression in ovariectomized rats tested 2 wk postovariectomy and rats tested 6 wk postovariectomy, which had gained >50 g more weight. This has two implications. First, it suggests that estradiol's effects on CCK-induced c-Fos expression are independent of body weight and adiposity. If estradiol interacts with adiposity signals, this interaction apparently fails to affect CCK-induced c-Fos expression. One study suggests that leptin insensitivity may be involved in the hyperphagia and body weight gain after ovariectomy (1), but other studies have failed to reveal changes in exogenous leptin's effect on food intake after ovariectomy (13, 48) or across the estrous cycle of gonadally intact rats (21). Our finding of similar expression of c-Fos in ovariectomized rats before and after significant body weight gain is consistent with these reports that estradiol does not interact with leptin to control meal size in female rats. Second, the independence of CCK-induced c-Fos and body weight suggests that the effect of exogenous leptin on exogenous CCK-induced c-Fos expression (23) does not reflect a similar interaction between endogenous leptin and exogenous CCK.

### Perspectives

We report here that estradiol treatment increased CCK-induced c-Fos expression in the cNTS, the spNTS, the iNTS, the PVN, and the CeA. Because CCK apparently mediates a major part of the estrogenic inhibition of feeding, which is expressed as decreased meal size (7, 27), these data suggest that estradiol affects neural activity in a widely distributed neural network controlling satiation. It is not yet possible to say what specific contribution to the process of satiation is made by any of these areas or what the relationships among the neural activities in different areas are. Because CCK satiation depends on vagal afferents (54) and because vagal afferents project to the NTS, the most parsimonious hypothesis appears to be that estradiol increases the responsiveness of NTS neurons that are postsynaptic to vagal afferents encoding CCK satiation and that neurons in other brain areas in which

activity is increased are downstream from these. This could occur whether estradiol acts locally in the NTS or acts in some other brain site containing ER- $\alpha$  that directly or indirectly projects to the NTS, such as the PVN or CeA. The latter alternative, that estradiol acts in the hypothalamus or limbic forebrain, would seem more in line with the known mechanisms of estradiol's actions on ovarian cycling and sexual receptivity. The former alternative, that estradiol acts in the NTS to affect feeding, is especially interesting in relation to the demonstrations by Grill and colleagues (31–33) that leptin, melanocortin-3/4 receptor agonists, urocortin, and other treatments that are usually assumed to act in the hypothalamus to affect feeding in fact have potent feeding effects when delivered locally to the fourth ventricle or NTS/dorsal vagal complex.

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### REFERENCES

1. Ainslie DA, Morris MJ, Wittert G, Turnbull H, Proietto J, and Thornburn AW. Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y. *Int J Obes Relat Metab Disord* 25: 1680–1688, 2001.
2. American Physiological Society. Guiding principles for research involving animals and human beings. *Am J Physiol Regul Integr Comp Physiol* 283: R281–R283, 2002.
3. Asarian L and Geary N. Cyclic estradiol treatment phasically potentiates endogenous cholecystokinin's satiating action in ovariectomized rats. *Peptides* 20: 445–450, 1999.
4. Asarian L and Geary N. Estradiol increases the satiating potency of intraduodenal infusions of intralipid, but not of L-phenylalanine, in ovariectomized rats. *Soc Neurosci Abstr* 25: 1556, 1999.
5. Asarian L and Geary N. Cyclic estradiol treatment normalizes body weight and restores physiological patterns of spontaneous feeding and sexual receptivity in ovariectomized rats. *Horm Behav* In press.
6. Barrachina MD, Martinez V, Wang L, Wei JY, and Tache Y. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci USA* 94: 10455–10460, 1997.
7. Blaustein JD and Wade GN. Ovarian influences on the meal patterns of female rats. *Physiol Behav* 17: 201–208, 1976.
8. Blessing WW. *The Lower Brainstem and Bodily Homeostasis*. New York: Oxford, 1997.
9. Butera PC, Bradway DM, and Cataldo NJ. Modulation of the satiety effect of cholecystokinin by estradiol. *Physiol Behav* 53: 1235–1238, 1993.
10. Butera PC, Willard DM, and Raymond SA. Effects of PVN lesions on the responsiveness of female rats to estradiol. *Brain Res* 576: 304–310, 1992.
11. Butera PC, Xiong M, Davis RJ, and Platania SP. Central implants of dilute estradiol enhance the satiety effect of CCK-8. *Behav Neurosci* 110: 823–830, 1996.
12. Chen DY, Deutsch JA, Gonzalez MF, and Gu Y. The induction and suppression of c-fos expression in the rat brain by cholecystokinin and its antagonist L364,718. *Neurosci Lett* 149: 91–94, 1993.
13. Chen Y and Heiman ML. Increased weight gain after ovariectomy is not a consequence of leptin resistance. *Am J Physiol Endocrinol Metab* 280: E315–E322, 2001.
14. Dagnault A and Richard D. Lesions of hypothalamic paraventricular nuclei do not prevent the effect of estradiol on energy and fat balance. *Am J Physiol Endocrinol Metab* 267: E32–E38, 1994.

15. **Dagnault A and Richard D.** Involvement of the medial preoptic area in the anorectic action of estrogens. *Am J Physiol Regul Integr Comp Physiol* 272: R311–R317, 1997.
16. **DiNardo LA and Travers JB.** Distribution of Fos-like immunoreactivity in the medullary reticular formation of the rat after gustatory elicited ingestion and rejection behaviors. *J Neurosci* 17: 3826–3839, 1997.
17. **Drewett RF.** The meal patterns of the oestrous cycle and their motivational significance. *Q J Exp Psychol B* 26: 489–494, 1974.
18. **Eckel LA and Geary N.** Endogenous cholecystokinin's satiating action increases during estrus in female rats. *Peptides* 20: 451–456, 1999.
19. **Eckel LA and Geary N.** Estradiol treatment increases feeding-induced c-Fos expression in the brains of ovariectomized rats. *Am J Physiol Regul Integr Comp Physiol* 281: R738–R746, 2001.
20. **Eckel LA, Houtp TA, and Geary N.** Spontaneous meal patterns in female rats with and without access to running wheels. *Physiol Behav* 70: 397–405, 2000.
21. **Eckel LA, Langhans W, Kahler A, Campfield LA, Smith FJ, and Geary N.** Chronic administration of OB protein decreases food intake by selectively reducing meal size in female rats. *Am J Physiol Regul Integr Comp Physiol* 275: R186–R193, 1998.
22. **Emond M, Ladenheim EE, Schwartz GJ, Moran TH.** Leptin amplifies the feeding inhibition and neural activation arising from a gastric nutrient preload. *Physiol Behav* 72: 123–128, 2001.
23. **Emond MH, Schwartz GJ, Ladenheim EE, and Moran TH.** Central leptin modulates behavioral and neural responsiveness to CCK. *Am J Physiol Regul Integr Comp Physiol* 276: R1545–R1549, 1999.
24. **Flanagan-Cato LM, King JL, Blechman JG, and O'Brien MP.** Estrogen reduces cholecystokinin-induced c-Fos expression in the rat brain. *Neuroendocrinology* 67: 384–391, 1998.
25. **Friedman JM.** Leptin and the regulation of body weight. *Harvey Lect* 95: 107–136, 2000.
26. **Geary N.** Estradiol, CCK and satiation. *Peptides* 22: 1251–1263, 2001.
27. **Geary N and Asarian L.** Cyclic estradiol treatment normalizes body weight and test meal size in ovariectomized rats. *Physiol Behav* 67: 141–147, 1999.
28. **Geary N, Asarian L, Korach KS, Pfaff DW, and Ogawa N.** Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. *Endocrinology* 142: 4751–4757, 2001.
29. **Geary N, Trace D, McEwen B, and Smith GP.** Cyclic estradiol replacement increases the satiety effect of CCK-8 in ovariectomized rats. *Physiol Behav* 56: 281–289, 1994.
30. **Glatzle J, Kreis ME, Kawano K, Raybould HE, and Zittel TT.** Postprandial neuronal activation in the nucleus of the solitary tract is partly mediated by CCK-A receptors. *Am J Physiol Regul Integr Comp Physiol* 281: R222–R229, 2001.
31. **Grill HJ and Kaplan JM.** The neuroanatomical axis for control of energy balance. *Front Neuroendocrinol* 23: 2–40, 2002.
32. **Grill HJ, Markison S, Ginsberg A, and Kaplan JM.** Long-term effects on feeding and body weight after stimulation of forebrain or hindbrain CRH receptors with urocortin. *Brain Res* 867: 19–28, 2000.
33. **Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breinger J, and Baskin DG.** Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 143: 239–246, 2002.
34. **Houtp TA.** CREB phosphorylation in the nucleus of the solitary tract and parabrachial nucleus is not altered by peripheral cholecystokinin that induces c-Fos. *Brain Res* 751: 143–147, 1997.
35. **Hrupka BJ, Smith GP, and Geary N.** Ovariectomy and estradiol affect postingestive controls of sucrose licking. *Physiol Behav* 61: 243–247, 1997.
36. **Hrupka BJ, Smith GP, and Geary N.** Central implants of dilute estradiol fail to reduce feeding in ovariectomized rats. *Physiol Behav* In press.
37. **Laviano A, Meguid MM, Gleason JR, Yang ZJ, and Renzville T.** Comparison of long-term feeding pattern between male and female Fischer 344 rats: influence of estrous cycle. *Am J Physiol Regul Integr Comp Physiol* 270: R413–R419, 1996.
38. **Li BH and Rowland NE.** Cholecystokinin- and dexfenfluramine-induced anorexia compared using devazepide and c-fos expression in the rat brain. *Regul Pept* 50: 223–233, 1994.
39. **Lindén A, Uvnäs-Moberg K, Forsberg G, Bednar I, and Södersten P.** Involvement of cholecystokinin in food intake: III. Oestradiol potentiates the inhibitory effect of cholecystokinin octapeptide on food intake in ovariectomized rats. *J Neuroendocrinol* 2: 797–801, 1990.
40. **Luckman SM.** Fos-like immunoreactivity in the brainstem of the rat following peripheral administration of cholecystokinin. *J Neuroendocrinol* 4: 149–152, 1992.
41. **Matson CA, Reid DF, and Ritter RC.** Daily CCK injection enhances reduction of body weight by chronic intracerebroventricular leptin infusion. *Am J Physiol Regul Integr Comp Physiol* 282: R1368–R1373, 2002.
42. **Matson CA, Wiater MF, Kuijper JL, and Weigle DS.** Synergy between leptin and cholecystokinin (CCK) to control daily caloric intake. *Peptides* 18: 1275–1278, 1997.
43. **McCarthy MM, Coirini H, Schumaker M, Pfaff DW, McEwen BS, and Schwartz-Giblin S.** Ovarian steroid modulation of [<sup>3</sup>H]muscimol binding in the spinal cord of the rat. *Brain Res* 556: 321–323, 1991.
44. **Murphy M, Barranco J, and Eckel LA.** Time course of estradiol's inhibitory effect on meal size in female rats. *Appetite Abstr* 37: 32, 2001.
45. **Norgren R.** The gustatory system. In: *The Rat Nervous System*, edited by Paxinos G. San Diego: Academic, 1995, p. 751–771.
46. **Olson BR, Hoffman GE, Sved AF, Stricker EM, and Verbalis JG.** Cholecystokinin induces c-fos expression in hypothalamic oxytocinergic neurons projecting to the dorsal vagal complex. *Brain Res* 569: 238–248, 1992.
47. **Paxinos G and Watson C.** *The Rat Brain in Stereotaxic Coordinates*. San Diego, CA: Academic, 1998.
48. **Pelleymounter MA, Baker MB, and McCaleb M.** Does estradiol mediate leptin's effects on adiposity and body weight? *Am J Physiol Endocrinol Metab* 277: E955–E963, 1999.
49. **Riedy CA, Chavez M, Figlewicz DP, and Woods SC.** Central insulin enhances sensitivity to cholecystokinin. *Physiol Behav* 58: 755–760, 1995.
50. **Rinaman L, Baker EA, Hoffman GE, Stricker EM, and Verbalis JG.** Medullary c-Fos activation in rats after ingestion of a satiating meal. *Am J Physiol Regul Integr Comp Physiol* 275: R262–R268, 1998.
51. **Rinaman L, Hoffman GE, Dohanics J, Le WW, Stricker EM, and Verbalis JG.** Cholecystokinin activates catecholaminergic neurons in the caudal medulla that innervate the paraventricular nucleus of the hypothalamus in rats. *J Comp Neurol* 360: 246–256, 1995.
52. **Rinaman L, Verbalis JG, Stricker EM, and Hoffman GE.** Distribution and neurochemical phenotypes of caudal medullary neurons activated to express cFos following peripheral administration of cholecystokinin. *J Comp Neurol* 338: 475–490, 1993.
53. **Schumaker M, Coirini H, Pfaff DW, and McEwen B.** Light-dark differences in behavioral sensitivity to oxytocin. *Behav Neurosci* 105: 487–492, 1991.
54. **Schwartz GJ and Moran TH.** Integrative gastrointestinal actions of the brain-gut peptide cholecystokinin in satiety. In: *Progress in Psychobiology and Physiological Psychology*, edited by Fluharty SJ and Morrison A. New York: Academic, 1998, p. 1–34.
55. **Shughrue PJ, Lane MV, and Merchenthaler I.** Comparative distribution of estrogen receptor- $\alpha$  and - $\beta$  mRNA in the rat central nervous system. *J Comp Neurol* 388: 507–525, 1997.
56. **Smith GP and Gibbs J.** The development and proof of the CCK hypothesis of satiety. In: *Multiple Cholecystokinin Receptors in the CNS*, edited by Dourish CT, Cooper SJ, Iversen SD, and Iversen LL. Oxford: Oxford University Press, 1992, p. 166–182.
57. **Wade GN, Gray JM, and Bartness TJ.** Gonadal influences on adiposity. *Int J Obes* 9: 83–92, 1985.
58. **Woods SC, Schwartz MW, Baskin DG, and Seeley RJ.** Food intake and the regulation of body weight. *Annu Rev Psychol* 51: 255–277, 2000.