Neuronal activation in the hypothalamus and brainstem during feeding in rats

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Summary

We trained rats to a regime of scheduled feeding, in which food was available for only 2 hr each day. After 10 days, rats were euthanized at defined times relative to food availability, and their brains were analyzed to map Fos expression in neuronal populations to test the hypothesis that some populations are activated by hunger whereas others are activated by satiety signals. Fos expression accompanied feeding in several hypothalamic and brainstem nuclei. Food ingestion was critical for Fos expression in noradrenergic and non-noradrenergic cells in the nucleus tractus solitarii and area postrema and in the supraoptic nucleus, as well as in melanocortin-containing cells of the arcuate nucleus. However, anticipation of food alone activated other neurons in the arcuate nucleus and in the lateral and ventromedial hypothalamus, including orexin neurons. Thus orexigenic populations are strongly and rapidly activated at the onset of food presentation, followed rapidly by activity in anorexigenic populations when food is ingested.

Introduction

Classically, the neural regulation of feeding involves an alternation between "hunger" signals, including those arising from the gastrointestinal tract, which activate hunger centers in the hypothalamus, and "satiety" signals, also arising from the gastrointestinal tract, which activate satiety centers. The hunger and the satiety centers interact with each other and are modulated by signals reporting on stored energy and metabolic status. They are also sensitive to environmental cues, such as those arising from photoperiod.

In particular, energy intake and utilization is regulated by several discrete subpopulations of neurons in the hypothalamus and brainstem. These populations are interconnected, and they express several different neuropeptides that have orexigenic or anorexigenic effects when injected centrally. Neurons in the arcuate nucleus that produce neuropeptide Y (NPY) are particularly important. NPY is a potent orexigen, the arcuate NPY neurons are activated by circulating ghrelin and inhibited by circulating leptin (Cowley et al., 2003; van den Top et al., 2004), and in mice, selective destruction of these neurons produces anorexia (Gropp et al., 2005; Luquet et al., 2005; Bewick et al., 2005). Within the arcuate nucleus, the NPY neurons innervate pro-opiomelanocortin (POMC) neurons (Cowley et al., 2001). This projection is inhibitory and is mediated mainly by GABA, which is coexpressed with NPY (Ovesjo et al., 2001). The POMC neurons produce the anorectic peptides α-melanocyte-stimulating hormone (α-MSH) and cocaine- and amphetamine-related peptide (CART); the anorexic effects of α-MSH are mediated by hypothalamic MC4 receptors, and genetic variants of the MC4 receptor are linked to obesity in humans (Govaerts et al., 2005). The arcuate NPY neurons also synthesize agouti-related protein (AgRP), which is an endogenous antagonist at the MC4 receptor (Ollmann et al., 1997). The POMC neurons are activated by leptin and inhibited by ghrelin, and these effects are both direct and indirect via the NPY/AgRP neurons (Cone, 2005).

Both the POMC cells and the NPY cells project extensively within the hypothalamus, especially to the lateral hypothalamus (LH), the ventromedial hypothalamus (VMH), the paraventricular nucleus (PVN), and the dorsomedial hypothalamus (DMH) (Bagnol et al., 1999). The LH is a "hunger center" that contains neurons that express orexins and melanin-concentrating hormone (MCH); lesions of the LH result in anorexia. The VMH is a "satiety center" and includes neurons that are glucose responsive (Ono et al., 1987); lesions of the VMH result in hyperphagia. The DMH also contains some orexin neurons and NPY neurons, it projects to the PVN, and it densely expresses MC4 receptors. DMH lesions block food entrainment of locomotor activity, body temperature, and wakefulness (Gooley et al., 2006). The PVN contains parvocellular neurons that express thyrotropin-releasing hormone and corticotrophin-releasing factor (CRF), which are important regulators of energy utilization, as well as magnocellular oxytocin and vasopressin neurons that regulate natriuresis and antidiuresis. The supraoptic nucleus (SON) also contains magnocellular oxytocin and vasopressin neurons, but no parvocellular neurons. The PVN and the SON express abundant MC4 receptors (Mountjoy et al., 1994) and are innervated by projections from arcuate POMC neurons (Bagnol et al., 1999).

These hypothalamic populations are regulated by signals from the brainstem that mediate signals from the gut, whereas projections from the PVN to the brainstem regulate gastric reflexes. Among the ascending projections, noradrenergic neurons of the nucleus tractus solitarii (NTS) are activated by gastric distension and by peripheral cholecystokinin (CCK), and some of these neurons project directly to magnocellular oxytocin neurons of the SON and PVN (Onaka et al., 1995) and to parvocellular neurons in the PVN. Several peptides are coexpressed in subpopulations of the noradrenergic neurons, and these

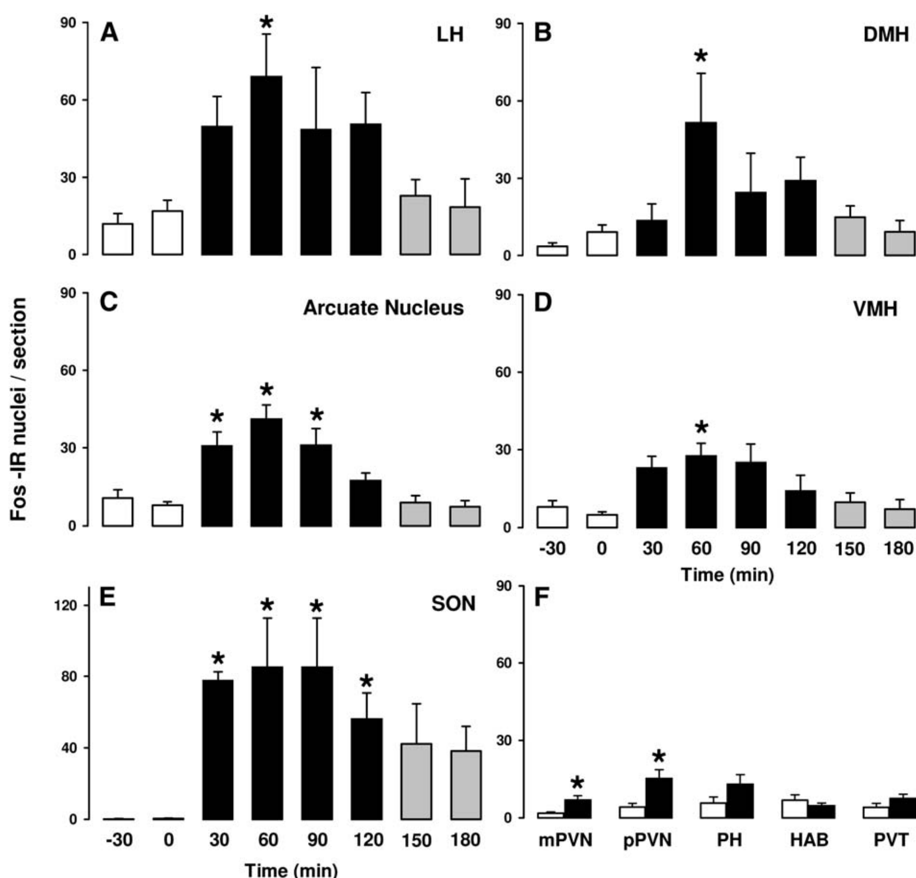

Figure 1. Temporal pattern of Fos expression

(A–E) Fos expression in the hypothalamus of rats before feeding, (−30 min [6 rats], 0 min [5 rats]; open bars); during food presentation (30 min [6 rats], 60 min [6 rats], 90 min [6 rats], 120 min [7 rats]; black bars); and after feeding (150 min [7 rats] and 180 min [7 rats]; gray bars). Fos+ cells were counted in 6–8 sections/area, and rat group means (± SE) were calculated for the following areas: LH (A) DMH (B), arcuate (C), VMH (D), and SON (E) (* indicates $p < 0.05$, versus 0 min).

(F) There were few Fos+ cells in the mPVN, pPVN, PH, HAB, and PVT, so data at −30 and 0 min (open bars) and at 60 and 90 min were pooled (black bars) (* indicates $p < 0.05$, versus prefeeding controls, Mann-Whitney rank sum test).

neurons are also functionally diverse (Simonian and Herbison, 1997). Other peptidergic neurons in the NTS that do not express noradrenaline also project to the hypothalamus, and some also carry feeding-related signals that arise from the gut (Luckman and Lawrence, 2003). Blood-borne signals related to nutrient status are also detected in the area postrema (AP), a circumventricular organ adjacent to the NTS that is outside the blood-brain barrier (Yamamoto et al., 2003).

In these experiments, we tried to establish the sequence of activation of specific neuronal populations during feeding. Many neurons express the immediate-early gene *c-fos* when activated (Morgan and Curran, 1991). Fos, the protein transcription-factor product of *c-fos*, is expressed within 30–60 min of induction of *c-fos* and can be quantified within a brain area or an identified neuronal population by counting cells that contain Fos. Accordingly, we trained rats to a regime of scheduled feeding, in which food was available for only 2 hr each day (rats were fed during the light period to avoid the effect of photoperiodic cue). The body weight of rats initially fell, but remained stable after about 5 days. After 10 days, rats were euthanized at defined times relative to food availability, and their brains were analyzed to map Fos expression in feeding-related neuronal populations. Some rats were also euthanized without being fed at the expected time. The activated populations were investigated by double immunohistochemistry for Fos and each of the following: CART, oxytocin, MCH, α-MSH, β-endorphin, orexin A, and tyrosine hydroxylase (TH, the rate-limiting enzyme in noradrenaline synthesis).

Results

Rats were trained to expect food for just 2 hr per day. After 10 days, food intake and body weight were stable, and as soon as food was presented, rats began to eat simultaneously and voraciously. After about 90 min, they stopped eating although food was still available, consistent with acute satiety.

Before feeding (times −30 and 0 min), there was little Fos in most areas examined, and no significant differences between −30 min and 0 min (Figure 1). By contrast, during food presentation (30, 60, 90 and 120 min), there was extensive Fos expression in many brain regions; generally, expression was high at 30 min, maximal at 60 or 90 min (Figure 1), and declined thereafter. In the LH, DMH, VMH, and arcuate nucleus, there were few Fos+ cells before feeding (Figures 1A–1D), but many at 30, 60, and 90 min (Figures 1A–1D). For example, in the LH, significantly more Fos was expressed at 60 min than at 0 min (ANOVA on ranks: $H = 22.40$, 7 degrees of freedom (df), $p = 0.002$; Dunn's Method, $p < 0.05$, versus 0 min). There were smaller, but significant increases in expression in the magnocellular PVN (mPVN) and pPVN; in these areas, there were no significant differences on pairwise group comparisons, but there was significantly more expression in the pooled data from 60 and 90 min than before feeding ($p < 0.05$, Figure 1F). By contrast to the mPVN, there was intense Fos expression in the supraoptic nucleus (SON) during feeding (Figure 1E; all comparisons $p < 0.05$, versus 0 min); at 30, 60, and 90 min, most SON neurons expressed Fos. There were no significant differences in Fos expression in

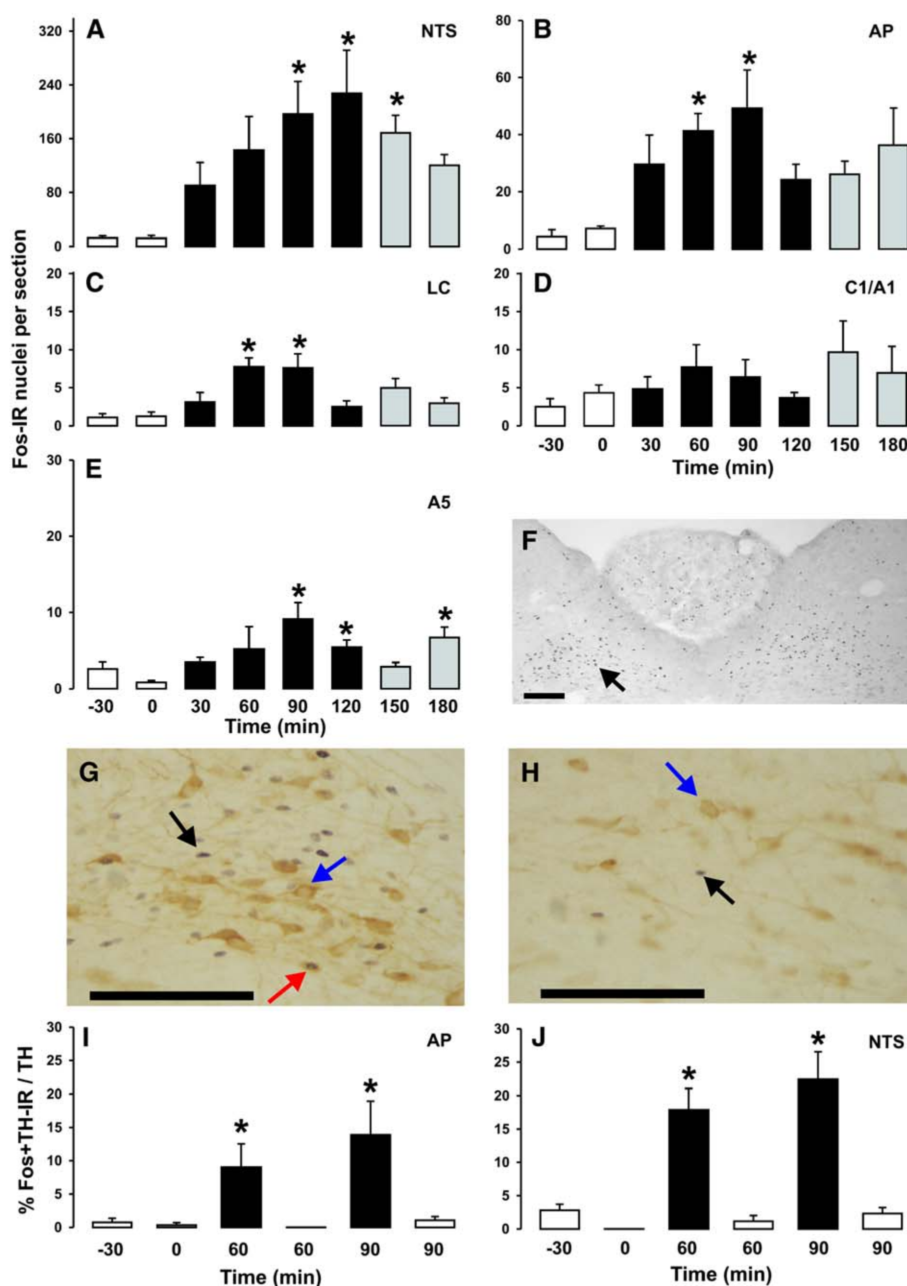

Figure 2. Effect of scheduled feeding on Fos expression in the caudal brainstem

(A–E) Mean (±SE) Fos+ cells per 40 μm section from rats before feeding (−30 min [6 rats], 0 min [6 rats]; open bars), after food presentation (30 min [6 rats], 60 [6 rats], 90 [6 rats], 120 min [7 rats]; black bars), and after feeding (150 min [7 rats], 180 min [6 rats]; gray bars). Statistical analyses for the NTS (A), AP (B), LC (C), C1/A1 (D), and A5 regions (E) (* $p < 0.05$, versus 0 min, Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method) are shown.

(F) Fos+ cells in the AP and NTS of a fed rat (60 min). Scale bar represents 100 μm.

(G and H) NTS cells labeled for Fos and TH in fed rats (60 min, [G]) and unfed rats (60 min after scheduled feeding time, [H]). Black arrow indicates Fos+ nuclei (black). Blue arrow indicates TH+ cytoplasm (brown). Red arrow indicates Fos+/TH+ cell; scale bars represent 50 μm.

(I and J) Fos+/TH+ cells as % of TH+ cells in the AP (I) and NTS (J) before (−30, 0 min, open bars) and during (60, 90 min, black bars) feeding and in unfed rats (60, 90 min after scheduled feeding time, open bars).

other brain areas in the same sections, i.e., the posterior hypothalamus (PH), habenular nucleus (HAB), and paraventricular nucleus of the thalamus (PVT; Figure 1F).

In the caudal brainstem, there were few Fos+ cells before feeding in the NTS, area postrema (AP), locus coeruleus (LC), and the C1/A1 and A5 regions (Figures 2A–2E). During feeding, Fos expression was increased markedly and significantly in the NTS and AP at 60 and 90 min ($p < 0.05$, Figures 2A, 2B, and 2F), and there was a small but significant increase in the LC (Figures 2A–2C) and in the A5 region at 90, 120, and 180 min (Figure 2E; $p < 0.05$, versus 0 min). There were no significant changes in Fos expression in the C1/A1 region at any time (Figure 2D).

Anticipatory activation of Fos expression

Two groups of six rats were euthanized at 60 and 90 min after the scheduled food presentation, but without being fed ("unfed rats"). Data from these 12 rats were pooled and compared with data from 12 fed rats euthanized at the same times, and with pooled data for the two prefeeding groups (controls, n = 11; Figures 3A and 3B). In the SON, mPVN, AP, NTS, and LC, Fos expression in unfed rats was similar to that in controls, and less than in fed rats ($p < 0.05$, Figures 3A and 3B). Food ingestion induced a striking amount of expression in the SON ($p < 0.05$, Figure 3D) in comparison to controls (Figure 3C) and unfed rats (Figure 3E), and this remained high at 150 min (Figure 3F). In the arcuate, pPVN, VMH, and LH, Fos expression was similar in fed and unfed rats, and higher than in controls (Figure 3A). In the PH (Figure 3A) and PVT, expression was low in unfed rats, fed rats, and controls (PVT: 4.5 ± 1.2, 7.5 ± 1.6, 6.8 ± 2.0 cells/section, respectively), but in the HAB, there was significantly more Fos in unfed rats (17.1 ± 4.9, $p < 0.05$) than in fed rats (7.5 ± 1.6) or controls (3.9 ± 1.3).

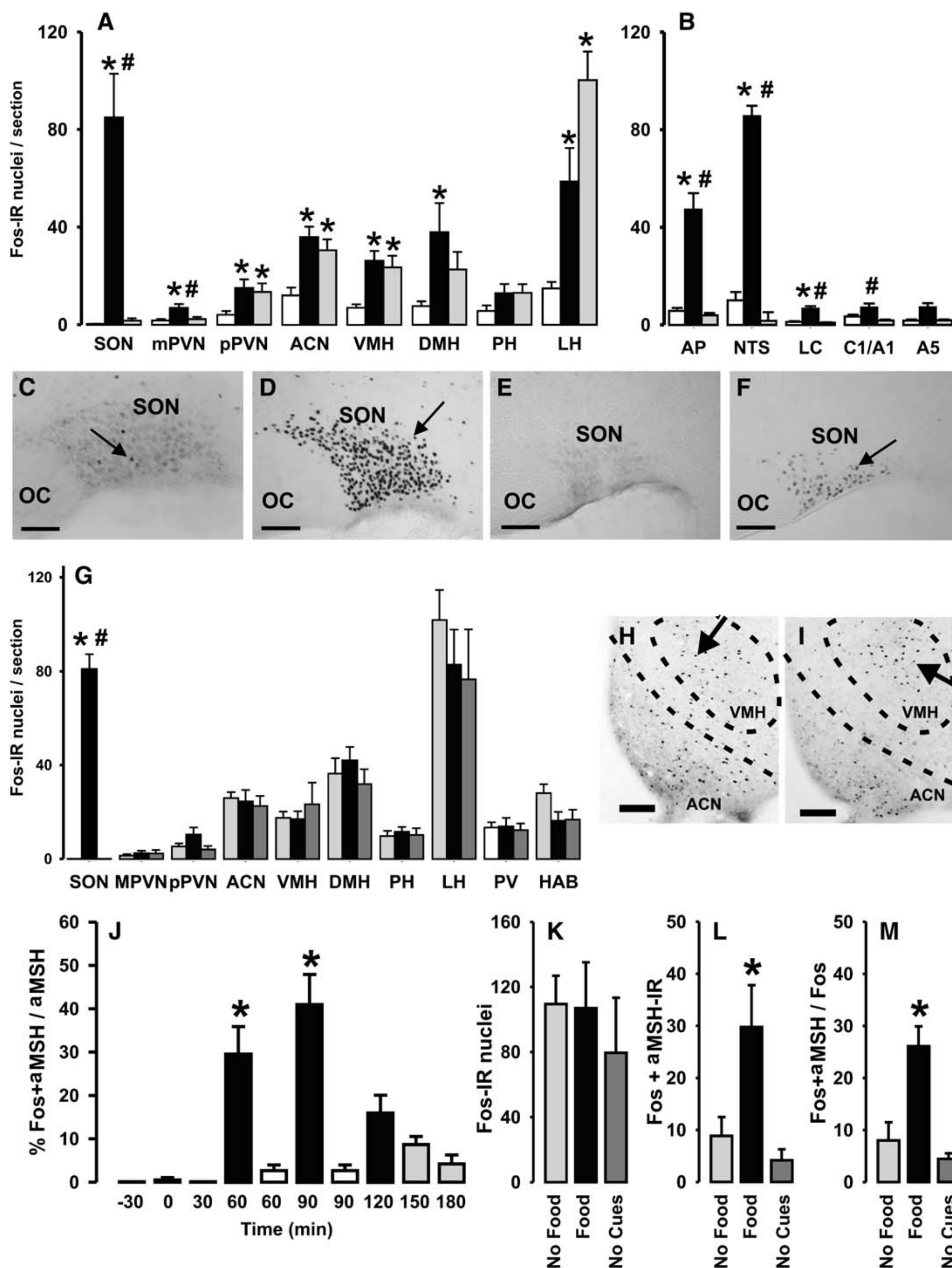

Figure 3. Effect of food ingestion and food cues on Fos expression

(A and B) Mean (±SE) Fos+ cells/section in rats before (−30, 0 min pooled; open bars) and during feeding (60, 90 min pooled; black bars) or in unfed rats (60, 90 min after scheduled feeding time, pooled; gray bars). * indicates $p < 0.05$, versus prefeeding; # indicates $p < 0.05$, versus unfed rats (Kruskal-Wallis ANOVA on ranks, Dunn's method, 10–12 rats/group). The number of Fos+ cells in the NTS is per section; for the AP, LC, C1/A1, and A5 regions, left and right counts are combined. ACN denotes arcuate nucleus.

(C–F) Fos in the SON at 0 min (C), 60 min (D), and 60 min unfed (E). (F) shows Fos+ cells in the SON after feeding (150 min). OC denotes optic chiasm; arrows indicate Fos+ nuclei; scale bars represent 100 μm.

(G) Fos at 60 min after scheduled feeding time; six rats were fed (black bars), six were in the same room but not fed (light gray bars), and six in another room and not fed (dark gray bars). * indicates $p < 0.05$, versus unfed rats in the same room, and # indicates $p < 0.05$, versus unfed rats in another room.

(H and I) Fos in the arcuate and VMH of a fed (H) and an unfed rat in another room (I).

(J) Fos+/α-MSH+ cells as a % of α-MSH+ cells in control rats (−30, 0 min), unfed rats (60, 90 min; open bars), fed rats (60, 90, 120 min; black bars), and after feeding (150, 180 min; gray bars). * indicates $p < 0.05$, versus 0 min (Dunn's method).

(K–M) Fos+ cells (K), Fos+/α-MSH+ cells (L), and Fos+/α-MSH+ cells as a % of Fos+ cells (M) at 60 min after scheduled feeding time. * indicates $p < 0.05$ (Dunn's method, all-pairwise).

In the suprachiasmatic nucleus (SCN), Fos expression was more variable than in any other area counted, and there were no significant differences between groups. Before feeding (0, −30 min), there were 36 ± 11 Fos+ cells/section (range 11–109, n = 11); in fed rats (60, 90 min) there were 29 ± 14, (2–134, n = 12), and in unfed rats (60, 90 min), there were 49 ± 9 (11–101, n = 10). Thus we found no evidence of acute changes in activity of the SCN at the time of feeding.

The effect of cues (e.g., the sound of other rats receiving and eating food) was studied in 18 rats (270 ± 2 g). Six rats were euthanized at 60 min during feeding, and six rats in the same room and six rats undisturbed in a different room were euthanized at the same time but not fed (unfed rats and "no-cues" group). There were no significant differences in Fos expression between groups in the mPVN, pPVN, arcuate, VMH, DMH, or LH (Figure 3G). Again, the SON showed dense Fos expression in fed rats ($p < 0.05$), but not in the other groups. We also counted Fos+ cells for the unfed, fed, and no-cues groups in the caudate putamen (37.8 ± 8.3, 20.1 ± 8.9, 47.3 ± 23.1 cells/section), nucleus accumbens shell (9.6 ± 2.4, 12.9 ± 3.8, 10.3 ± 3.9), and nucleus accumbens core (7.2 ± 2.0, 4.8 ± 1.7, 5.2 ± 2.3); there were no significant differences between groups in any of these areas ($p > 0.05$).

Although the number of Fos+ cells in the arcuate was not significantly different between fed and unfed rats, whether in the same or a different room (Figures 3H and 3I), the distribution of expression differed. Fed rats expressed Fos in both the medial and lateral parts of the nucleus, whereas Fos+ cells in unfed rats were mainly in the medial part (Figures 3H and 3I). We did not quantify this difference because it was recognized only post hoc, but instead interrogated it by double immunocytochemistry.

Characterization of activated neurons

In the LH, during feeding, many orexin-A+ cells contained Fos (Figures 4A and 4B). There were significantly more Fos+/orexin+ cells at 30 (22.1 ± 5.2 cells/section), 60 (22.5 ± 5.7), and 90 min (18.3 ± 3.9) than at 0 min (4.9 ± 3.6; all $p < 0.05$, versus 0 min). The number of Fos+/orexin+ cells at 150 min (7.5 ± 1.8) was not significantly different from that at 0 min. Sections from three rats at 60 min were double stained for MCH (Figure 4C), and 12% of MCH+ cells expressed Fos (153 of 1651 cells). There were few orexin+ cells in the DMH, so double labeling was not quantified in this area. In the PVT, orexin and NPY were present in fibers but not cell bodies.

In the SON, double labeling confirmed that much of the Fos was in magnocellular oxytocin cells (Figures 4H–4J). In the PVN, there was little Fos expression, and double labeling was not quantified, but Fos was seen in occasional parvocellular and magnocellular oxytocin cells (Figure 4L).

In the arcuate, POMC cells express CART, β-endorphin, and α-MSH, but it is not clear to what extent these are differentially expressed. Some sections from all rats were studied by double labeling for Fos and α-MSH. Labeling for Fos and either CART or β-endorphin was performed on sections from some rats only; data from unfed rats with and without cues are too few for meaningful comparisons. Groups are combined to give large enough n values for meaningful comparisons where appropriate.

Before feeding, few arcuate cells contained both Fos and α-MSH (Figure 3J), but many Fos+ cells were colocalized with α-MSH during feeding (Figures 4K and 3J). The number of Fos+/α-MSH+ cells significantly increased with time through 60 and 90 min ($p < 0.05$), then decreased to prefeeding levels by 180 min (Figure 3J). At 60 min, 28% of Fos+ cells contained α-MSH (299 of 1050 Fos+ cells in 27 sections from 11 rats, making up 27% of 1096 α-MSH+ cells; Figure 3M). However, in unfed rats (Figure 3K) there was little coexpression of Fos and α-MSH, whether or not cues were present (Figures 4L, 3L, and 3M).

Before feeding, few arcuate cells contained both Fos and β-endorphin (e.g., in eight sections from two rats at 0 min, 24 of 781 β-endorphin+ cells contained Fos). However, during feeding, many Fos+ cells contained β-endorphin; thus, in ten sections from two rats at 90 min, 115 of 843 β-endorphin+ cells contained Fos, i.e., 43% of all Fos was colocalized with β-endorphin. By 150 min, (30 min after removing food), very few β-endorphin+ cells expressed Fos (23 of 1060 in two rats).

Similarly, before feeding, few arcuate cells contained both Fos and CART (−30, 0 min combined, 5.6 ± 1.6 cells/section, four rats), but many Fos+ cells were colocalized with CART during feeding (Figure 4M: 60, 90 min combined, 15.3 ± 2.5, nine rats). Similar colocalization was seen in unfed rats (60, 90 min combined, 12.7 ± 3.0 cells/section, six rats) and in rats after feeding (120, 150 min combined, 14.7 ± 1.7, five rats). Thus, in the arcuate nucleus of fed rats, Fos was expressed to a similar extent in cells immunoreactive for CART, α-MSH, and β-endorphin. Surprisingly, in unfed rats, Fos was expressed in CART+ cells but not in α-MSH+ cells.

The involvement of noradrenergic neurons was investigated by double labeling for Fos and TH. Before feeding, few cells contained both Fos and TH, but during feeding many Fos+ cells contained TH in the NTS and AP ($p < 0.05$, Figures 2G–2J).

Discussion

Several studies have examined the effects of orexigenic and anorexigenic factors on Fos expression in the hypothalamus and caudal brainstem (e.g., Hewson and Dickson, 2000; Zittel et al., 1999; Zheng, et al., 2002; Brown et al., 1998), but the temporal profile of Fos expression over a bout of feeding has not been described. In the present study, rats were accustomed to expect food for just 2 hr per day. After 10 days, their body weight and food intake were stable, and when given food they ate promptly and voraciously. After about 90 min, they stopped eating although food was still available, consistent with acute satiety. We had expected there to be a clear temporal dissociation between brain regions activated by hunger, which would peak at the scheduled time of food presentation, and regions activated when the rats stopped eating. Instead, neurons that release orexigenic peptides appear to be activated by the imminent expectation of food, and neurons implicated in satiety are activated as soon as any food is eaten.

At 30 min after food presentation, Fos expression was increased in the LH, arcuate nucleus, SON, and VMH. This is close to the minimum latency after induction of *c-fos* mRNA expression, and it indicates strong neuronal activation coincident with the onset of feeding. Notably, the areas activated include areas thought to mediate satiety as well as areas thought to mediate hunger. In particular, feeding resulted in rapid activation in the arcuate, LH, DMH, VMH, and SON, and in the NTS and AP. Significant but less extensive activation was also seen in other areas, including the PVN. In the arcuate, LH, DMH, and VMH,

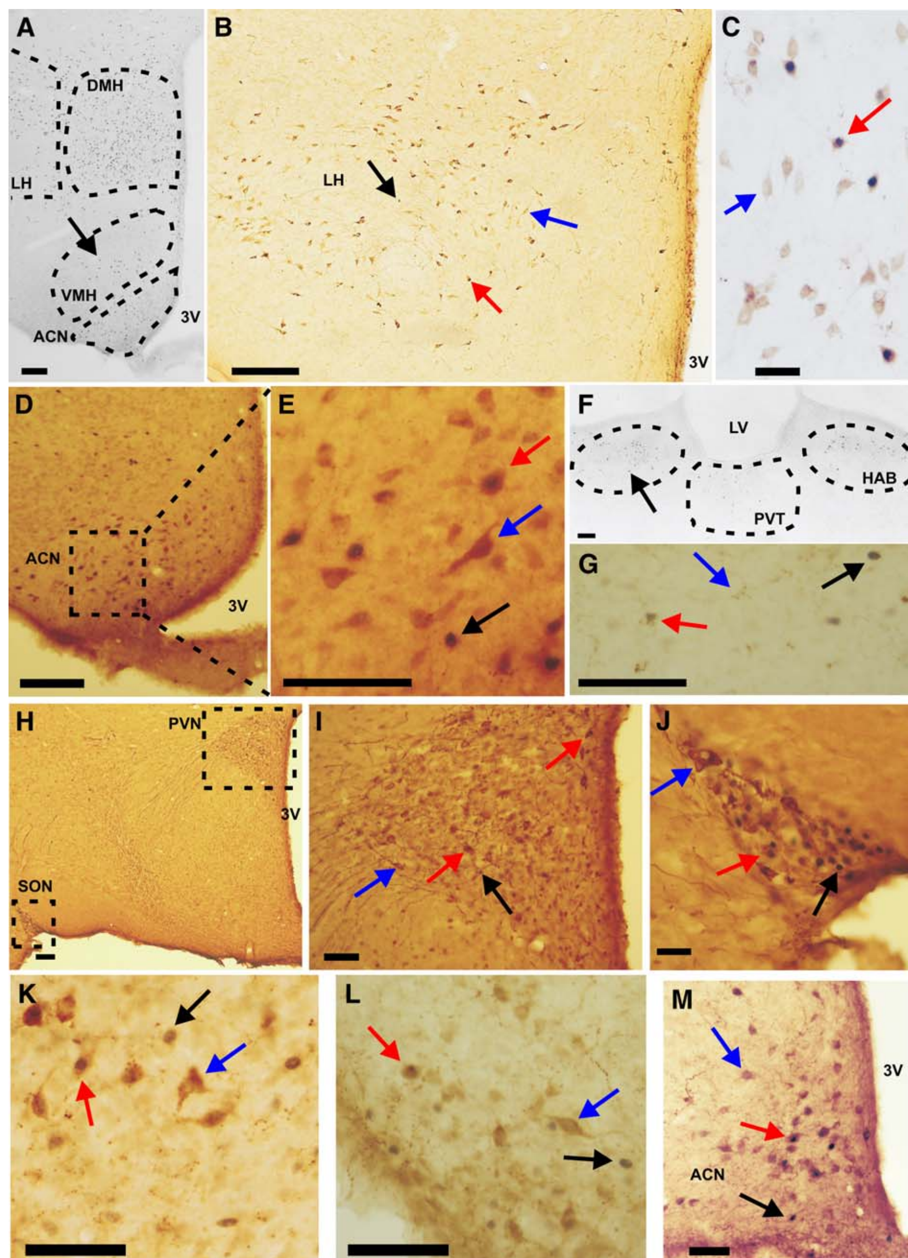

Figure 4. Neurochemical nature of food-activated neurons

Immunohistochemistry in brains of fed rats (A–K, M) or unfed rats (L) at 60 min after scheduled feeding time. Fos+ nuclei (black arrows) are black; cytoplasmic peptides are brown (blue arrows). Red arrows indicate the double-IR cells.

(A and B) Fos and orexin A in the LH (A) and at higher magnification (B).

(C) Fos and MCH in the LH.

(D and E) Fos and β-endorphin in the arcuate (D) and at higher magnification (E).

(F) Fos in the PVT and HAB.

(G) Fos and orexin in the PVT. In the PVT, orexin is in fiber varicosities close to Fos+ cells.

(H–J) Low magnification (H) of Fos and oxytocin in the PVN (I) and SON (J). There are many Fos+ cells in the SON but few in the PVN.

(K and L) Fos and α-MSH in the arcuate of a fed rat at 60 min (K) and an unfed rat at the same time (L); there are fewer Fos+/α-MSH+ cells in the unfed rat.

(M) Fos and CART in the arcuate.

Scale bars represent 100 μm in (A), (D), (F), (H)–(J), and (M) and 50 μm in (B), (C), (E), (G), (K), and (L).

Fos expression was induced at the expected time of food presentation even in the absence of food, but in the SON, NTS, and AP, Fos was expressed only in fed rats. In the LH, Fos was expressed in orexin A neurons and in MCH neurons, and in the NTS in TH neurons. Both orexin and MCH potently increase food intake when injected centrally (Stanley et al., 2005).

The gross anatomical map of Fos expression might be misleading. Although the arcuate nucleus showed a similar level of Fos expression in unfed rats as in fed rats, fed rats contained more α-MSH+/Fos+ cells, indicating that although some arcuate neurons are activated in anticipation of food, α-MSH cells are activated only when food is eaten. Most of the arcuate neurons that showed anticipatory Fos expression were in the ventromedial part of the nucleus, and they probably include NPY neurons. Ghrelin agonists activate Fos expression in NPY cells in this region (Dickson and Luckman, 1997). Unfortunately, cytoplasmic NPY staining was too weak and fiber staining too intense for unequivocal identification of Fos+ cells (data not shown). α-MSH is coexpressed with CART and β-endorphin, which is also a product of POMC (Appleyard et al., 2003). Surprisingly, therefore, CART cells but not α-MSH cells were activated in unfed rats. More than 90% of arcuate CART cells express POMC, but, except in the retrochiasmatic part of the nucleus, only about half of POMC cells express CART (Elias et al., 1998). The processing of POMC to α-MSH is poorly understood (Pritchard et al., 2002), and it is possible that in the arcuate nucleus CART neurons and α-MSH neurons are functionally separate.

Other studies have shown anticipatory effects of scheduled feeding, including increased locomotor activity before feeding and increased Fos expression in brain areas. Nakahara et al. (2004) found increased Fos expression before feeding in the PVT, but not in other brain areas. Meynard et al. (2005) found

activation of histamine neurons of the tuberomammillary nucleus in anticipation of feeding, and of perifornical orexin cells during feeding. Angeles-Castellanos et al. (2004) reported high Fos expression at mealtime in schedule-fed rats, including in the DMH, LH, PVN, and the tuberomammillary nucleus, with no change in the SCN, in broad agreement with the present results. Ablation of the SCN does not inhibit food-anticipatory behaviors in schedule-fed rats (Mistlberger, 1994).

Food ingestion induced Fos expression in the SON, mPVN, NTS, AP, and (to a lesser extent) the LC and A5 regions. The AP is a site at which blood-borne factors are recognized—e.g., glucagon-like peptide I (Yamamoto et al., 2003) and PYY (Deng et al., 2001)—and projects to the adjacent NTS, which receives ascending information from the afferent gastric vagus; the NTS is a major source of projections to the hypothalamus, and these include A2 noradrenergic cells and several populations of peptidergic neurons. Among these, cells that express prolactin-releasing peptide (Roland et al., 1999; Luckman and Lawrence, 2003) and neuromedin U (Ivanov et al., 2004) are implicated in the control of feeding. The NTS also contains cells that respond to systemic ghrelin (Li et al., 2006), and POMC cells of unknown function (Cone, 2005).

Some A2 cells project directly to oxytocin cells in the SON and mPVN. This pathway is activated by gastric distension and by intravenous (i.v.) CCK (Monnikes et al., 1997), resulting in oxytocin release from the posterior pituitary and consequent natriuresis (Leng et al., 1999). Vasopressin cells in the SON were also active during feeding, but the pathway by which these are activated is not clear; they are not innervated by A2 cells, and are inhibited by i.v. CCK. Oxytocin and vasopressin secretion during feeding probably minimizes disturbances in electrolyte homeostasis that would otherwise accompany ingestion of solutes. Although activation of oxytocin cells is an expected consequence of activation of the NTS, the SON receives an olfactory bulb input that may also be active during feeding, and this innervates the SON but not the PVN (Hatton, 1990), which might explain the selective activation of the SON seen here. A similar differential response was reported by Chaillou et al. (2000): Fos was expressed in the SON of fed but not unfed sheep, but in other brain areas they saw no difference between fed and unfed sheep, or more expression in unfed sheep, as was the case in the PVN.

Fos expression often reflects increased electrical activity, but not always. In particular, α-MSH injected centrally induces Fos expression in oxytocin cells of the SON, but inhibits their electrical activity (Sabatier et al., 2003). α-MSH acts on these cells via MC4 receptors to mobilize intracellular Ca^{2+} stores, leading to production of endocannabinoids that presynaptically suppress afferent inputs (Sabatier and Leng, 2006), thereby suppressing both electrical activity and peripheral oxytocin secretion. Meanwhile, the intracellular Ca^{2+} signal triggers oxytocin release from the neuronal dendrites, and hence α-MSH suppresses peripheral oxytocin secretion while activating central release. Thus although Fos expression indicates a change in neuronal activity, what type of change needs further study. Finally, lack of Fos expression is weak evidence for lack of a change, and we cannot exclude the involvement of brain areas where we did not detect a change in Fos expression.

In conclusion, when rats are trained to expect food for 2 hr per day, hypothalamic cells that express orexigenic peptides express Fos at the scheduled time of food presentation, even if food is not presented. Cells that express anorexigenic peptides also express Fos at the time of food presentation, but only if food is presented. Thus "satiety" circuits are activated coincidentally with food intake, rather than only after a threshold intake is exceeded.

Experimental Procedures

Male Sprague-Dawley rats were housed singly at 21°C–22°C in a 12 hr/12 hr light/dark cycle (lights on at 07:00, off at 19:00), fed standard rat chow, and given free access to water. All procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986.

Scheduled feeding

Rats were weighed at 08:00 and given food and water at 08:00 and 18:00. Rats (332 ± 15 g, n = 30) fed ad libitum ate 30.9 ± 0.9 g/day over 5 days (26.1 ± 0.7 g in the dark, 4.8 ± 0.4 g in the light) and drank 41.7 ± 1.2 ml water/day (36.6 ± 1.1 ml in the dark, 7.5 ± 0.6 ml in the light), accompanied by an increase in body weight of 13.4 ± 1.7 g over 5 days. The effect of scheduled feeding for 10 days (food for 2 hr/day: 09:00–11:00) was tested in a pilot study of 16 rats (419 ± 14 g on day 0). Food intake was less on day 1 (3.2 ± 0.5 g, $p < 0.05$) than on any other day, but was stable with no significant differences on days 2–10 (range 7.5 ± 0.4 to 11.0 ± 2.0 g), with a daily food intake of ~36% of normal. Body weight declined to 355 ± 14 g on days 6 and 7 ($p < 0.05$), but was stable thereafter. When food was presented, the rats immediately began to eat and continued to eat for about 90 min. We previously measured the expression of CRF and vasopressin mRNA in the parvocellular PVN (pPVN), and NPY mRNA in the arcuate nucleus of rats on this feeding schedule; NPY mRNA expression was high, but CRF and vasopressin mRNA expression levels were normal (Johnstone et al., 2005), indicating that the rats are not chronically stressed, as also found by others (Wilkinson et al., 1979).

Temporal pattern of Fos expression

Rats were euthanized after 10 days of schedule feeding; at 30 min before feeding; at 0 min (09:00, the scheduled feeding time); and at 30, 60, 90, 120, 150, and 180 min after food presentation. In a separate experiment, rats were fed at 09:00 (fed rats), while others in the same room were not (unfed rats); these were euthanized at 60 or 90 min after the scheduled time of feeding. Other rats in a separate room had no food and no sound of food being presented (no-cues group), and were euthanized at 60 min after the scheduled feeding time.

Rats were euthanized with sodium pentobarbitone (1 ml, 60 mg/ml, intraperitoneal [i.p.]) and rapidly perfuse fixed with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were rapidly removed and postfixed (0.1 M PB, 4% paraformaldehyde, 30% sucrose) for 24 hr, followed by 15% sucrose in 0.1 M PB for 24 hr, then frozen and stored at −70°C. Coronal sections (40 μm) were cut on a microtome from 1.6 mm anterior to bregma to 4.2 mm posterior (Paxinos and Watson, 1996), and from 9.7 to 14.3 mm posterior to bregma, and stored in cryoprotectant (30% ethylene glycol, 20% glycerol, 50% 0.2 M PBS) until processing for Fos immunoreactivity (IR). Cryoprotectant was removed by washing in 0.1 M PB, and endogenous peroxidase was deactivated with 20% methanol, 0.03% hydrogen peroxidase in 0.1 M PB. The sections were incubated in *c-fos* anti-rabbit antibody (Ab-2, Oncogene Biosciences) diluted at 1:1000, in 0.1 M PB containing 1% normal sheep serum (NSS) and 0.02% Triton-X (T: Sigma) for 48 hr at 4°C. After washing, sections were incubated in biotinylated anti-rabbit IgG in 0.1 M PB (1:400, Vectastain ABC Elite kit: Vector) for 1 hr at room temperature and then, after further washing, for 1 hr in avidin-biotinylated horseradish peroxidase. After washing, Fos-IR was visualized by the nickel-diaminobenzidine (DAB)-glucose oxidase method (Shu et al., 1988). Sections for Fos-IR only were mounted on gelatinized slides and were then dehydrated through a series of alcohols followed by xylene, and coverslipped with DePex (BDH).

For double immunohistochemistry (IHC), sections processed for Fos were washed and incubated in rabbit anti-α-MSH (Peninsula Laboratories) at 1:400 in 0.1 M PBT containing 2% NSS; rabbit anti-β-endorphin (Peninsula) at 1:5000 in 1% NSS in 0.1 M PBT; rabbit anti- CART(61-102) (Phoenix Pharmaceuticals) at 1:4000 in 2% NSS in 0.1 M PBT; rabbit anti-oxytocin

(Peninsula) at 1:1000 in 2% NSS in 0.1 M PBT; rabbit polyclonal anti-orexin A 14-33 (Ab-2: Oncogene) at 1:4000 in 2% NSS in 0.1 M PBT; rabbit anti-MCH (Phoenix) at 1:2000 in 1% NSS in 0.1 M PBT; and rabbit anti-neuropeptide Y (Ab-1, Oncogene) at 1:1500 in 1% NSS in 0.1 M PBT. Secondary antibodies were detected by Vectastain ABC Elite kit (Rabbit: Pk-6100, Vector) and visualized in 0.33% hydrogen peroxide, 0.25% DAB in 0.1 M PB. Monoclonal mouse anti-TH (Chemicon: 1:1000 in 0.1 M PBT containing 2% normal horse serum, NHS) followed by biotinylated anti-mouse (1:500 in 0.1 M PBT, 2% NHS) and streptavidin-biotinylated horseradish peroxidase complex (1:200 in 0.1 M PBT, 1% NHS) was used to detect noradrenergic cells. Sections were mounted on slides, dehydrated through a series of alcohols, and coverslipped with DePeX.

Fos positive (+) nuclei appeared black and colocalized peptides as brown-stained cytoplasm. Sections were viewed under a light microscope, and their distance from bregma was estimated by reference to Paxinos and Watson (1996). For sections labeled for Fos only, areas were examined with 10× objective (Leica). Fos+ cells were counted bilaterally in six to eight sections per rat at regular intervals throughout each area. Double-stained sections were examined with 20× objective, and Fos+ cells, cytoplasmic-IR cells, and double-labeled cells were counted. Mean counts per section of the bilateral paired nuclei were calculated for each rat, followed by group means (±SE). Counts were made blind on coded slides.

Statistics

Changes in food intake and body weight of rats were compared by repeated measures ANOVA on ranks followed by Holm-Sidak method (versus day 1). Differences in Fos expression were analyzed by one-way ANOVA followed by Student-Newman-Keuls method (for normally distributed data) or Kruskal-Wallis ANOVA on ranks followed by Dunn's method (for nonnormally distributed data). Pairwise comparisons were with the control time (0 min) except where stated otherwise. Post-hoc analysis was only performed when group differences were significant by ANOVA at $p < 0.05$.

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References

Angeles-Castellanos, M., Aguilar-Roblero, R., and Escobar, C. (2004). c-Fos expression in hypothalamic nuclei of food-entrained rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. *286*, R158–R165. Published online August 21, 2003. 10.1152/ajpregu.00216.2003.

Appleyard, S.M., Hayward, M., Young, J.I., Butler, A.A., Cone, R.D., Rubinstein, M., and Low, M.J. (2003). A role for the endogenous opioid beta-endorphin in energy homeostasis. Endocrinology *144*, 1753–1760.

Bagnol, D., Lu, X.Y., Kaelin, C.B., Day, H.E., Ollmann, M., Gantz, I., Akil, H., Barsh, G.S., and Watson, S.J. (1999). Anatomy of an endogenous antagonist: Relationship between Agouti-related protein and proopiomelanocortin in brain. J. Neurosci. *19*, RC26.

Bewick, G.A., Gardiner, J.V., Dhillo, W.S., Kent, A.S., White, N.E., Webster, Z., Ghatei, M.A., and Bloom, S.R. (2005). Post-embryonic ablation of AgRP neurons in mice leads to a lean, hypophagic phenotype. FASEB J. *19*, 1680–1682.

Brown, K.S., Gentry, R.M., and Rowland, N.E. (1998). Central injection in rats of alpha-melanocyte-stimulating hormone analog: Effects on food intake and brain Fos. Regul. Pept. *78*, 89–94.

Chaillou, E., Baumont, R., Tramu, G., and Tillet, Y. (2000). Effect of feeding on Fos protein expression in sheep hypothalamus with special reference to the supraoptic and paraventricular nuclei: An immunohistochemical study. Eur. J. Neurosci. *12*, 4515–4524.

Cone, R.D. (2005). Anatomy and regulation of the central melanocortin system. Nat. Neurosci. *8*, 571–578.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature *411*, 480–484.

Cowley, M.A., Smith, R.G., Diano, S., Tschop, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., et al. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron *37*, 649–661.

Deng, X., Guarita, D.R., Pedroso, M.R., Kreiss, C., Wood, P.G., Sved, A.F., and Whitcomb, D.C. (2001). PYY inhibits CCK-stimulated pancreatic secretion through the area postrema in unanesthetized rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. *281*, R645–R653.

Dickson, S.L., and Luckman, S.M. (1997). Induction of c-fos messenger ribonucleic acid in neuropeptide Y and growth hormone (GH)-releasing factor neurons in the rat arcuate nucleus following systemic injection of the GH secretagogue, GH-releasing peptide-6. Endocrinology *138*, 771–777.

Elias, C.F., Lee, C., Kelly, J., Aschkenasi, C., Ahima, R.S., Couceyro, P.R., Kuhar, M.J., Saper, C.B., and Elmquist, J.K. (1998). Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron *21*, 1375–1385.

Gooley, J.J., Schomer, A., and Saper, C.B. (2006). The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. Nat. Neurosci. *9*, 398–407.

Govaerts, C., Srinivasan, S., Shapiro, A., Zhang, S., Picard, F., Clement, K., Lubrano-Berthelier, C., and Vaisse, C. (2005). Obesity-associated mutations in the melanocortin 4 receptor provide novel insights into its function. Peptides *26*, 1909–1919.

Gropp, E., Shanabrough, M., Borok, E., Xu, A.W., Janoschek, R., Buch, T., Plum, L., Balthasar, N., Hampel, B., Waisman, A., et al. (2005). Agouti-related peptide-expressing neurons are mandatory for feeding. Nat. Neurosci. *8*, 1289–1291.

Hatton, G.I. (1990). Emerging concepts of structure-function dynamics in adult brain: The hypothalamo-neurohypophysial system. Prog. Neurobiol. *34*, 437–504.

Hewson, A.K., and Dickson, S.L. (2000). Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. J. Neuroendocrinol. *12*, 1047–1049.

Ivanov, T.R., Le Rouzic, P., Stanley, P.J., Ling, W.Y., Parello, R., and Luckman, S.M. (2004). Neuromedin U neurones in the rat nucleus of the tractus solitarius are catecholaminergic and respond to peripheral cholecystokinin. J. Neuroendocrinol. *16*, 612–619.

Johnstone, L.E., Srisawat, R., Kumarnsit, E., and Leng, G. (2005). Hypothalamic expression of NPY mRNA, vasopressin mRNA and CRF mRNA in response to food restriction and central administration of the orexigenic peptide GHRP-6. Stress *8*, 59–67.

Leng, G., Brown, C.H., and Russell, J.A. (1999). Physiological pathways regulating the activity of magnocellular neurosecretory cells. Prog. Neurobiol. *57*, 625–655.

Li, Y., Wu, X., Zhao, Y., Chen, S., and Owyang, C. (2006). Ghrelin acts on the dorsal vagal complex to stimulate pancreatic protein secretion. Am. J. Physiol. *290*, G1350–G1358. Published online February 9, 2006. 10.1152/ajpgi.00493.2005.

Luckman, S.M., and Lawrence, C.B. (2003). Anorectic brainstem peptides: More pieces to the puzzle. Trends Endocrinol. Metab. *14*, 60–65.

Luquet, S., Perez, F.A., Hnasko, T.S., and Palmiter, R.D. (2005). NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. Science *310*, 683–685.

Meynard, M.M., Valdes, J.L., Recabarren, M., Seron-Ferre, M., and Torrealba, F. (2005). Specific activation of histaminergic neurons during daily feeding anticipatory behavior in rats. Behav. Brain Res. *158*, 311–319.

Mistlberger, R.E. (1994). Circadian food-anticipatory activity: Formal models and physiological mechanisms. Neurosci. Biobehav. Rev. *18*, 171–195.

Monnikes, 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. *273*, R2059–R2071.

Morgan, J.I., and Curran, T. (1991). Stimulus-transcription coupling in the nervous system: Involvement of the inducible proto-oncogenes fos and jun. Annu. Rev. Neurosci. *14*, 421–451.

Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B., and Cone, R.D. (1994). Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol. Endocrinol. *8*, 1298–1308.

Nakahara, K., Fukui, K., and Murakami, N. (2004). Involvement of thalamic paraventricular nucleus in the anticipatory reaction under food restriction in the rat. J. Vet. Med. Sci. *66*, 1297–1300.

Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., and Barsh, G.S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science *278*, 135–138.

Onaka, T., Luckman, S.M., Antonijevic, I., Palmer, J.R., and Leng, G. (1995). Involvement of the noradrenergic afferents from the nucleus tractus solitarii to the supraoptic nucleus in oxytocin release after peripheral cholecystokinin octapeptide in the rat. Neuroscience *66*, 403–412.

Ono, T., Sasaki, K., and Shibata, R. (1987). Feeding- and chemical-related activity of ventromedial hypothalamic neurones in freely behaving rats. J. Physiol. *394*, 221–237.

Ovesjo, M.L., Gamstedt, M., Collin, M., and Meister, B. (2001). GABAergic nature of hypothalamic leptin target neurones in the ventromedial arcuate nucleus. J. Neuroendocrinol. *13*, 505–516.

Paxinos, G., and Watson, C. (1996). The Rat Brain in Stereotaxic Coordinates (Sydney: Academic Press).

Pritchard, L.E., Turnbull, A.V., and White, A. (2002). Pro-opiomelanocortin processing in the hypothalamus: Impact on melanocortin signalling and obesity. J. Endocrinol. *172*, 411–421.

Roland, B.L., Sutton, S.W., Wilson, S.J., Luo, L., Pyati, J., Huvar, R., Erlander, M.G., and Lovenberg, T.W. (1999). Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. Endocrinology *140*, 5736–5745.

Sabatier, N., and Leng, G. (2006). Presynaptic actions of endocannabinoids mediate {alpha}-MSH-induced inhibition of oxytocin cells. Am. J. Physiol. Regul. Integr. Comp. Physiol. *290*, R577–R584. Published online November 3, 2005. 10.1152/ajpregu.00667.2005.

Sabatier, N., Caquineau, C., Dayanithi, G., Bull, P., Douglas, A.J., Guan, X.M., Jiang, M., Van der Ploeg, L., and Leng, G. (2003). Alpha-melanocyte-stimulating hormone stimulates oxytocin release from the dendrites of hypothalamic neurons while inhibiting oxytocin release from their terminals in the neurohypophysis. J. Neurosci. *23*, 10351–10358.

Shu, S., Ju, G., and Fan, L. (1988). The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci. Lett. *85*, 169–171.

Simonian, S.X., and Herbison, A.E. (1997). Differential expression of estrogen receptor and neuropeptide Y by brainstem A1 and A2 noradrenaline neurons. Neuroscience *76*, 517–529.

Stanley, S., Wynne, K., McGowan, B., and Bloom, S. (2005). Hormonal regulation of food intake. Physiol. Rev. *85*, 1131–1158.

van den Top, M., Lee, K., Whyment, A.D., Blanks, A.M., and Spanswick, D. (2004). Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus. Nat. Neurosci. *7*, 493–494.

Wilkinson, C.W., Shinsako, J., and Dallman, M.F. (1979). Daily rhythms in adrenal responsiveness to adrenocorticotropin are determined primarily by the time of feeding in the rat. Endocrinology *104*, 350–359.

Yamamoto, H., Kishi, T., Lee, C.E., Choi, B.J., Fang, H., Hollenberg, A.N., Drucker, D.J., and Elmquist, J.K. (2003). Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. J. Neurosci. *23*, 2939–2946.

Zheng, H., Corkern, M.M., Crousillac, S.M., Patterson, L.M., Phifer, C.B., and Berthoud, H.R. (2002). Neurochemical phenotype of hypothalamic neurons showing Fos expression 23 h after intracranial AgRP. Am. J. Physiol. Regul. Integr. Comp. Physiol. *282*, R1773–R1781.

Zittel, T.T., Glatzle, J., Kreis, M.E., Starlinger, M., Eichner, M., Raybould, H.E., Becker, H.D., and Jehle, E.C. (1999). C-fos protein expression in the nucleus of the solitary tract correlates with cholecystokinin dose injected and food intake in rats. Brain Res. *846*, 1–11.