The Action of Leptin on Appetite-Regulating Cells in the Ovine Hypothalamus: Demonstration of Direct Action in the Absence of the Arcuate Nucleus

Yue Qi, Belinda A. Henry, Brian J. Oldfield, and Iain J. Clarke

Department of Physiology, Monash University, Clayton, Victoria 3800, Australia

It is widely accepted that leptin acts on first-order neurons in the arcuate nucleus (ARC) with information then relayed to other hypothalamic centers. However, the extent to which leptin mediates its central actions solely, or even primarily, via this route is unclear. We used a model of hypothalamo-pituitary disconnection (HPD) to determine whether leptin action on appetite-regulating systems requires the ARC. This surgical preparation eliminates the ARC. We measured effects of iv leptin to activate hypothalamic neurons (Fos labeling). In ARC-intact animals, leptin increased the percentage of Fos-positive melanocortin neurons and reduced percentages of Fos-positive neuropeptide Y neurons compared with saline-treated animals. HPD itself increased Fos labeling in the lateral hypothalamic area (LHA). Leptin influenced Fos labeling in the dorsomedial nucleus (DMH), ventromedial nucleus, and paraventricular nucleus (PVN) in HPD and normal animals, with effects on particular cell types varying. In the LHA and DMH, leptin decreased orexin cell activation in HPD and ARC-intact sheep. HPD abolished leptin-induced expression of Fos in melanin-concentrating hormone cells in the LHA and in CRH cells in the PVN. In contrast, HPD accentuated activation in oxytocin neurons. Our data from sheep with lesions encompassing the ARC do not suggest a primacy of action of leptin in this nucleus. We demonstrate that first order to second order signaling may not represent the predominant means by which leptin acts in the brain to generate integrated responses. We provide evidence that leptin exerts direct action on cells of the DMH, ventromedial nucleus, and PVN. **(*Endocrinology* 151: 2106–2116, 2010)**

Leptin is produced predominantly by white adipocytes (1) and acts on the central nervous system to reduce feeding and increase energy expenditure in various species including rodents (2, 3), sheep (4–6), and humans (7). A popular model of metabolic regulation is one that involves leptin action on first-order neurons in the arcuate nucleus (ARC) with relay of sensory information derived from adiposity and nutrient status to other centers of the hypothalamus including the dorsomedial nucleus (DMH), ventromedial nucleus (VMN), lateral hypothalamic area (LHA), and paraventricular nucleus (PVN) (reviewed in Refs. 8 and 9).

The ARC is uniquely positioned, because it lies partially outside the blood-brain barrier and therefore receives signals from blood, allowing direct passage of blood-borne signals to the relevant neurons (10, 11). Leptin acts on cells in the ARC to increase activity of proopiomelanocortin (POMC) cells and to inhibit neuropeptide Y (NPY) neurons (12). In rodents and humans, these leptin-responsive cells of the ARC have been shown to project to cells of the LHA that produce orexin and melanin-concentrating hormone (MCH) (13, 14). In addition, there is neuroanatomical evidence that the VMN and PVN receive input from the ARC in sheep (15, 16) and in rodent species (8, 9) and that peptides derived from cells of the ARC act within the VMN and the PVN (17–20). Leptin resistance may manifest in cells of the ARC, as demonstrated by discrete upregulation of the suppressor of cytokine signaling 3 (SOCS-3) and associated reduction in signal transducer

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-1283 Received October 30, 2009. Accepted February 22, 2010.
First Published Online March 22, 2010

For editorial see page 1975

Abbreviations: ARC, Arcuate nucleus; DMH, dorsomedial nucleus; HPD, hypothalamo-pituitary disconnection; LHA, lateral hypothalamic area; MCH, melanin-concentrating hormone; MSG, monosodium glutamate; PB, phosphate buffer; POMC, proopiomelanocortin; NPY, neuropeptide Y; PVN, paraventricular nucleus; SF-1, steroidogenic factor-1; VMN, ventromedial nucleus.

and activator of transcription (STAT) activation after leptin administration in obese mice (21). Combined, these anatomical and functional observations support the notion that the ARC is a primary site of leptin action and that the information that is assimilated here is promulgated to other centers within the hypothalamus that control food intake and expenditure of energy.

Notwithstanding the evidence that supports the above model, there is good reason to believe that leptin can also act directly on hypothalamic cells located outside the ARC. This would require transport of leptin across the blood-brain barrier possibly via an active transport system involving the short leptin-receptor isoform, ObRa, which has been identified in microvessels throughout the hypothalamus (22). The signaling form of the leptin receptor (ObRb) is highly expressed in cells of the DMH, VMN, and ventral premammillary hypothalamic nuclei, and moderate levels of expression are also seen in cells of the periventricular nucleus, the PVN, LHA, medial mammillary nucleus, and posterior hypothalamic nucleus in rodents (23, 24). In the ovine hypothalamus, an immunohistochemical study localized the receptor to cells in the periventricular nucleus, PVN, DMH, VMN, and ARC as well as in cells in the perifornical, anterior hypothalamic, LHA, and the zona incerta (25). Furthermore, there is evidence illustrating that leptin activates neurons in hypothalamic nuclei other than the ARC. In mice, microinjection of leptin directly into the VMN reduces food intake and activates steroidogenic factor-1 (SF-1) neurons (26, 27); specific deletion of the leptin receptor in SF-1 neurons of the VMH exacerbates weight gain in models of diet-induced obesity (28). In addition, leptin microinjection into a number of hypothalamic nuclei increases sympathetic nerve discharge in rats (29). Thus, leptin may act directly upon cells in hypothalamic nuclei beyond the ARC.

We have used an ovine model of hypothalamo-pituitary disconnection (HPD), which has been used previously to isolate the pituitary from the brain, thereby eliminating the secretion of anterior pituitary gland hormones and causing atrophy of the neural lobe (30). This surgical procedure not only removes neural input to the median eminence but also involves extirpation of the ARC (30). We used the HPD model to test the hypothesis that leptin action on appetite-regulating hypothalamic systems requires an intact ARC to transmit information to second-order neurons in the VMN, DMH, LHA, and PVN.

Materials and Methods

Animals and ethics

Adult Corriedale ewes (53–58 kg body weight) were maintained on pasture. All procedures and tissue collections were in accordance with the institutional guidelines, and previous approval of the Monash University School of Biomedical Sciences Animal Ethics Committee was obtained.

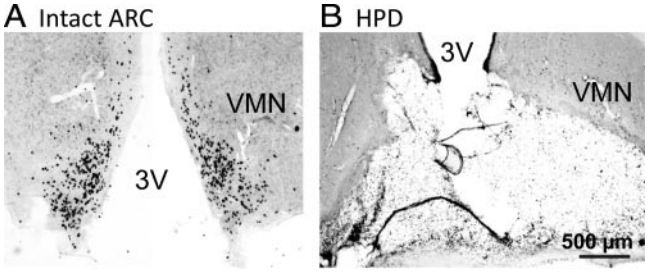

FIG. 1. Gray-scaled photomicrographs of the mediobasal hypothalamus of representative animals with intact ARC (A) and HPD (B), showing removal of the ARC in the latter. In the intact animal, POMC neurons were labeled with a digoxigenin-labeled POMC probe to help delineate the lateral margins of the ARC. In the HPD animal, the lesioned area is replaced by scar and granulation tissue. *Calibration bar* in B applies to both panels. 3V, Third ventricle.

HPD surgery and experiment design

HPD surgery was performed as originally described (30), although it was carried out in such a way to ensure that the ARC was completely removed (Fig. 1). Thus, after the separation of the median eminence from the basal hypothalamus, gentle suction was applied to the brain tissue of the medial periventricular region above the median eminence, and the extent to which removal of the ARC was achieved was verified histologically (*vide infra*). In brief, the surgical procedure was as follows. After anesthesia, the head was stabilized in a custom-made frame. The basal hypothalamus, median eminence, and pituitary gland were exposed by a transnasal, transsphenoidal route, and the dura mater was opened to allow access to the region immediately above the median eminence (30). A suction probe (1 mm) was introduced immediately above the vascular arcade on the anterior surface of the median eminence, and the ARC was removed by gentle suction, leaving the median eminence and pituitary separated from the hypothalamus. The operative tunnel was packed with gelatin sponge and sealed with dental acrylic, before the nasal cavity was closed. The animals were allowed to recover on pasture for 1 month before experimentation.

Adult Corriedale ewes of similar age (eight control and eight HPD) were introduced to single pens 2 d before the experiment with free access to lucerne chaff and water. The animals were allocated to four groups as follows: group 1, ARC-intact with saline treatment; group 2, ARC-intact with leptin treatment; group 3, HPD surgery and saline treatment; and group 4, HPD surgery and leptin treatment.

Because the HPD animals had atrophied ovaries, the control animals were ovariectomized 3 months before the experiment to ensure comparable gonadal status. We have shown previously (31) that HPD animals become obese, but this does not occur until at least 16 wk after the operation. Accordingly, we did not take blood samples to compare and contrast markers of metabolic status or body condition.

Each animal received a jugular venous cannula (Dwellcath; Tuta Laboratories, Lane Cove, New South Wales, Australia) on the day before the experimental treatment, so iv injections could be made with minimal disturbance. Manometer lines (Tuta Laboratories) were connected to the cannulae, allowing extension to

the back of the animals' pens. The sheep were injected with either saline as vehicle (10 ml) or 2 mg human recombinant leptin. The leptin was produced as previously described, and bioactivity was verified in an *in vitro* bioassay (4).

The animals were treated in pairs with either saline or leptin and killed 90 min later by iv injection of 20 ml sodium pentobarbital (Lethabarb; May & Baker, Melbourne, Victoria, Australia). An iv injection of 25,000 IU heparin was administered 5 min before the lethal injection, and the heads were perfused through the external carotid arteries with 2 liters heparinized (12,500 IU/liter) 0.9% saline followed by 1 liter 10% formalin (Merck, Kilsyth, Victoria, Australia) in 0.1 M phosphate buffer [PB (pH 7.4); 0.1 M Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$] and then 0.5 liter 10% buffered formalin containing 20% sucrose. Brains were removed from the calvarium, and blocks of the preoptic area/hypothalamus were dissected and allowed to sink in 30% sucrose for 4–5 d at 4 C. The blocks were frozen on powdered dry ice and kept at −20 C. Coronal sections (40 μm) were cut on a cryostat into six series, and the sections were stored in cryoprotectant at −20 C.

Histological verification of ARC lesions

To verify the extent of ARC lesions, gene expression for POMC in the ARC was examined by *in situ* hybridization (32). To do this, at least three sections representing the rostral, middle, and caudal extent of the ARC were selected. An antisense riboprobe was generated (33) and was validated for *in situ* hybridization in sheep (34). Free-floating sections were prehybridized and hybridized overnight at 58 C, and then the sections were washed in 2× sodium saline citrate, 0.1% Tween 20 at 58 C, followed by 0.1× sodium saline citrate, 0.1% Tween 20 at 58 C. The sections were rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween 20) and incubated for 2 h in maleic acid buffer containing 2% blocking reagent (10% goat serum and 2 mM levamisole). An alkaline phosphatase-conjugated sheep antidigoxigenin antibody (1:1000; Roche, Castle Hill, New South Wales, Australia) was added, and incubation was overnight at 4 C. The digoxigenin-labeled neurons were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate salts (Roche), after which the sections were washed in maleic acid buffer with 2 mM levamisole followed by alkaline buffer [0.1 M NaCl, 0.1 M Tris-HCl (pH 9.5), 0.1 M $MgCl_2$, 0.1% Tween 20, 2 mM levamisole].

Fos-labeling immunohistochemistry

Anatomically matched sections for each targeted nucleus were selected using an atlas of the ovine brain (35). Incubations were performed at room temperature with four 10-min washes in 0.05 M PBS (pH 7.4; 0.05 M Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$ and 0.9% saline) between each step. After 20 min incubation in 1% sodium borohydride (Sigma-Aldrich, Castle Hill, New South Wales, Australia), the sections were treated with 3% H_2O_2 (Merck) for 20 min to minimize the endogenous peroxidase activity and then with 5% normal horse serum and 0.3% Triton X-100) for 1 h. The sections were incubated in a rabbit primary α-Fos antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight and then sequentially with polyclonal biotinylated swine antirabbit IgG (1:200; Dako, Carpinteria, CA) and the ABC solution (1:200, Vectastain; Vector Laboratories, Burlingame, CA) for 1 h. The sections were then incubated in a chromogen solution (1 mg/ml 3,3′-diaminobenzidine, 0.02% ammonium nickel chloride, and 0.026% cobalt chloride in 0.1 M PB; Merck reagents) for 10 min before the addition of 30% H_2O_2 (1:25,000) for 6 min. The procedure resulted in a black nuclear stain, and the sections were mounted on gelatin-coated slides, dried overnight, dehydrated the next day, and coverslipped with dibutyl phthalate xylene. All sections were processed for the visualization of Fos in one run.

Double-labeling immunohistochemistry

Three matched sections for each animal were selected to determine the neurochemical nature of Fos-labeled neurons using a double-label immunohistochemical procedure. After Fos labeling as described above, the sections were then treated again with 5% normal goat serum and 0.3% Triton X-100 in 0.1 M PB for 1 h. The sections were then incubated overnight with primary antibodies (all at 1:2000 dilution) against the following peptides: NPY (courtesy of Dr. A. Lawrence, Monash University, Melbourne, Australia) (15), orexin (courtesy of Dr. T. Sakurai, Institute for Materials Research, Tohoku University, Sendai, Japan) (15), MCH (courtesy of Prof. W. Vale, Salk Institute, La Jolla, CA) (15), CRH (courtesy of Prof. W. Vale) (36), γ-MSH (1:2000; Antibodies Australia, Melbourne, Australia) (36), and oxytocin (1:2000; Antibodies Australia) (36).

The sections were then sequentially incubated first in biotinylated goat antirabbit/guinea pig IgG at 1:400 dilution (Vector) and then streptavidin-biotinylated horseradish peroxidase complex (1:500; Amersham Biosciences, Buckinghamshire, UK) for 1 h, respectively. To visualize the labeling of neurons, the sections were preincubated in a chromogen solution (1 mg/ml 3,3′-diaminobenzidine in 25 ml 0.1 M PB) for 10 min and 30% H_2O_2 (1:5000) for 2 min, giving a brown stain in the neurons of interest. Sections were mounted on gelatin-coated slides, dried overnight, dehydrated, and coverslipped with dibutyl phthalate xylene. A negative control (no antiserum) was performed with each set.

Retrograde labeling of cells in the ARC with FluoroGold injection in the LHA

We have reported projections from the ARC to the PVN and the VMN in the sheep (16, 36) but not projections from the ARC to the LHA. Because this is of relevance to the question as to whether cells of the LHA are second-order neurons receiving input from the ARC, we performed the relevant study. In particular, we determined the number of NPY and POMC cells in the ARC that are retrogradely labeled when a retrograde tracer (FluoroGold) is microinjected into the ARC. The details of the injection sites in the lateral hypothalamus of five animals are given in a recent publication (36). The POMC and NPY cells of the ARC were immunostained as above.

Photography and cell counts

The number of cells that showed Fos immunoreactivity were imaged using Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD). Bilateral counts were made from anatomically matched sections of each nucleus. To estimate the number of double-labeled neurons, images were captured and cells were counted manually. A double-labeled cell was deemed Fos positive when the brown cytoplasmic staining completely surrounded the Fos-stained nucleus. Photomicrographs were made with images captured on an AxioCam MR microscope (Carl Zeiss, Inc., North Ryde, Sydney, Australia) and were grouped into plates using Adobe PhotoShop CS3. Only brightness and contrast were adjusted.

TABLE 1. The effect of HPD on the number of cells in the hypothalamus

Group	n	Total number of cells			
		ORX	MCH	CRH	OT
ARC-intact saline	4	400 ± 34.0	1760 ± 160.7	559 ± 273.4	1172 ± 188.5
ARC-intact leptin	4	428 ± 35.4	2060 ± 195.9	756 ± 261.8	1144 ± 149.0
HPD saline	4	330 ± 51.3	2137 ± 170.1	1109 ± 342.5	443 ± 92.5[a]
HPD leptin	4	416 ± 67.5	2000 ± 143.8	861 ± 175.2	555 ± 91.2[a]

The table shows group means ± SEM for the total number of orexin (ORX), MCH, CRH, and oxytocin (OT) cells in three sections per animal in the relevant regions of the hypothalamus.

[a] $P < 0.01$ with both ARC-intact groups.

Statistics

Statistical analyses were performed with SPSS 15.0 (SPSS, Chicago, IL). The data were analyzed by single-factor and the least significant differences test for *post hoc* comparisons.

Results

HPD: ablation of the ARC

The HPD operation caused bilateral destruction of the ARC in all cases, and this region was replaced by granulation and scar tissue (Fig. 1). In two animals, the lesion encroached marginally on the medial-ventral portion of the VMN. The HPD operation did not affect the number of cells in the hypothalamus that immunostained for CRH, orexin, or MCH but reduced ($P < 0.01$) the total number of cells immunostained for oxytocin (parvocellular and magnocellular) (Table 1).

Fos labeling in the ARC

Fos labeling was observed in the ARC of the ARC-intact, leptin-treated animals (Fig. 2), with increased ($P < 0.01$) labeling in both the lateral and ventral regions of the nucleus compared with vehicle-treated animals (Fig. 3, A and B). As previously reported (15), NPY cells are seen in the medial and ventral regions of the nucleus, whereas POMC cells are in the lateral regions. Double-label immunohistochemistry showed that Fos labeling in NPY cells in leptin-treated animals was reduced ($P < 0.01$) when compared with their saline-treated counterparts but increased ($P < 0.01$) in POMC cells (Fig. 4, A and B; for examples of double immunolabeling, see Fig. 6).

Fos labeling in the LHA (orexin and MCH cells)

Ablation of the ARC increased the number of Fos-positive cells ($P < 0.01$) in the LHA (Figs. 3C and 5, A–E). Leptin treatment increased ($P < 0.05$) Fos labeling in the LHA of the ARC-intact animals compared with the saline-treated ARC-intact sheep. Leptin failed to increase Fos immunostaining beyond that attributed to ARC ablation (Fig. 3C). Double labeling was performed to determine the extent of involvement of orexin and MCH cells (Figs. 4, C and D, and 6). In HPD vehicle-treated animals, the percentage of orexin cells containing Fos was lower ($P < 0.01$), whereas the percentage of MCH cells containing Fos was higher ($P < 0.01$) compared with that of ARC-intact vehicle-treated animals. Leptin treatment reduced ($P < 0.01$) Fos expression in orexin cells irrespective of the status of the ARC. Leptin treatment, however, increased ($P < 0.01$) Fos expression in MCH cells in ARC-intact animals, but this effect was abolished in the HPD group.

Fos labeling in the VMN

There was no effect of HPD surgery on the number of Fos-positive neurons in the VMN (Figs. 3D and 5, K–O), but leptin treatment increased ($P < 0.01$) Fos levels in both HPD and ARC-intact animals.

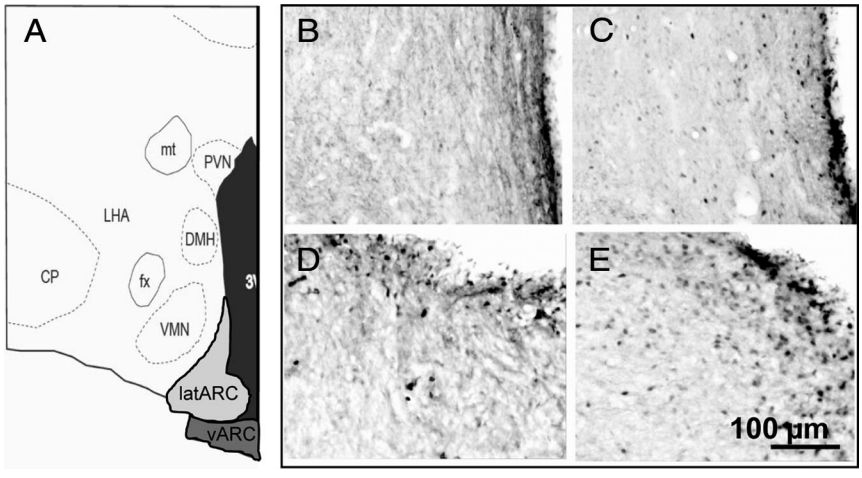

FIG. 2. Examples of Fos-immunostained neurons in the ARC of the ovine brain after leptin or vehicle treatment in ARC-intact animals. A, Schematic diagram illustrating the lateral (latARC) and ventral (vARC) regions of the ARC depicted in the grayscale photomicrographs (B–E). Representative photomicrographs are presented for the lateral ARC in saline-treated (B) and leptin-treated (C) animals as well as the ventral ARC in saline-treated (D) and leptin-treated (E) animals. fx, Fornix; mt, mammillothalamic tract; 3V, third ventricle.

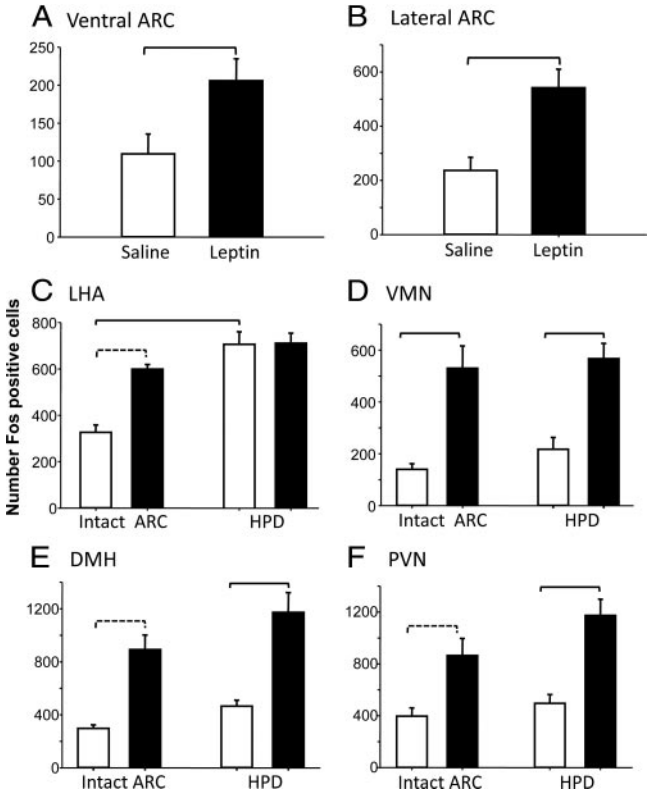

FIG. 3. The number of Fos-labeled neurons (mean ± SEM) in different hypothalamic nuclei after leptin (*black bars*) or vehicle (*white bars*) treatment of either HPD or ARC-intact animals. The *broken lines* indicate significant differences of $P < 0.05$; the *solid lines* indicate significant differences of $P < 0.01$.

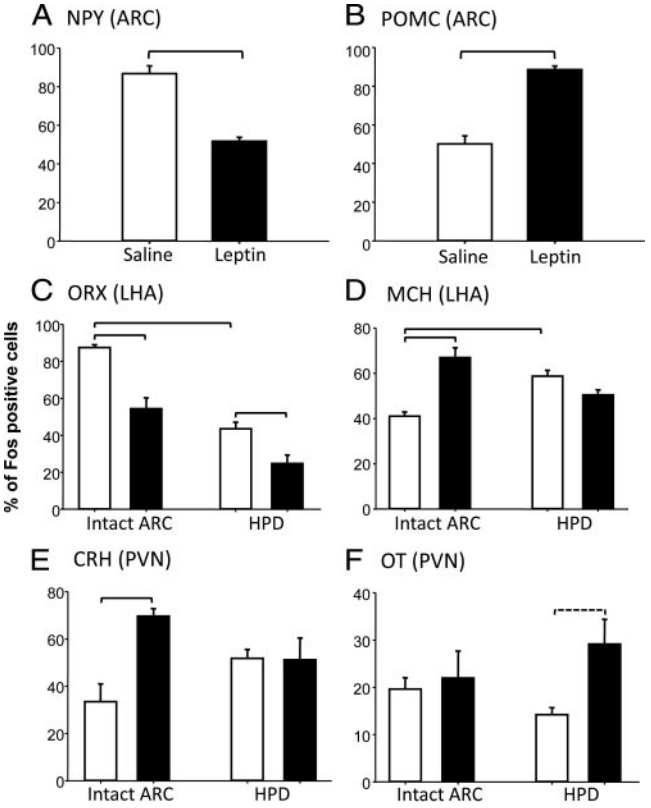

FIG. 4. The percentage of activated (Fos-positive) neurons (mean ± SEM) of particular neurochemical content after leptin (*black bars*) or vehicle (*white bars*) treatment in animals with an intact ARC or after HPD. The *broken line* indicates significant differences of $P < 0.05$; the *solid lines* indicate significant differences of $P < 0.01$. ORX, Orexin; OT, oxytocin.

Fos labeling in the DMH (orexin cells)

There was no effect of HPD surgery on the number of Fos-positive cells of the DMH (Figs. 3E and 5, F–J). Leptin treatment increased ($P < 0.01$) Fos labeling in the DMH of both ARC-intact and HPD sheep (Fig. 5K). Similar to the LHA, the percentage of orexin cells containing Fos was lower ($P < 0.01$) in HPD vehicle-treated animals compared with ARC-intact vehicle-treated controls (data not shown). Furthermore, leptin treatment reduced ($P < 0.01$) the percentage of orexin cells expressing Fos in both HPD and ARC-intact groups (data not shown).

Fos labeling in the PVN (CRH and oxytocin cells)

There was no effect of HPD surgery on the number of Fos-expressing cells in the PVN (Figs. 3F and 5, P–T). Leptin treatment increased Fos labeling in the PVN in both ARC-intact ($P < 0.05$) and HPD ($P < 0.01$) animals. We further detailed the effect in the PVN by examining Fos labeling in the parvocellular cells that express either CRH or oxytocin. Our study was restricted to the parvocellular cells because Fos immunostaining was confined to the region along the third ventricle, in which the parvocellular neurons are found (37). In ARC-intact animals, leptin treatment increased ($P < 0.01$) the percentage of CRH cells containing Fos, and this effect was abolished by HPD operation (Figs. 4E and 6). By comparison, there was a tendency to increase ($P < 0.1$) the percentage of leptin-responsive oxytocin neurons, and this effect was enhanced after HPD (Figs. 4F and 6).

Retrograde labeling of cells in the ARC with FluoroGold injection in the LHA

No POMC cells were retrogradely labeled in the ARC after FluoroGold injection in the lateral hypothalamus, and only a small percentage (2%) of NPY cells was retrogradely labeled (Table 2).

Discussion

We have employed a novel surgical approach to determine the extent to which the response of hypothalamic cells to leptin requires the presence of the ARC. Various cells of the ovine hypothalamus show a robust response to peripheral leptin measured by elevated or decreased levels of Fos expression. We demonstrate that responses of cells in the LHA to leptin are relayed by the ARC, whereas leptin

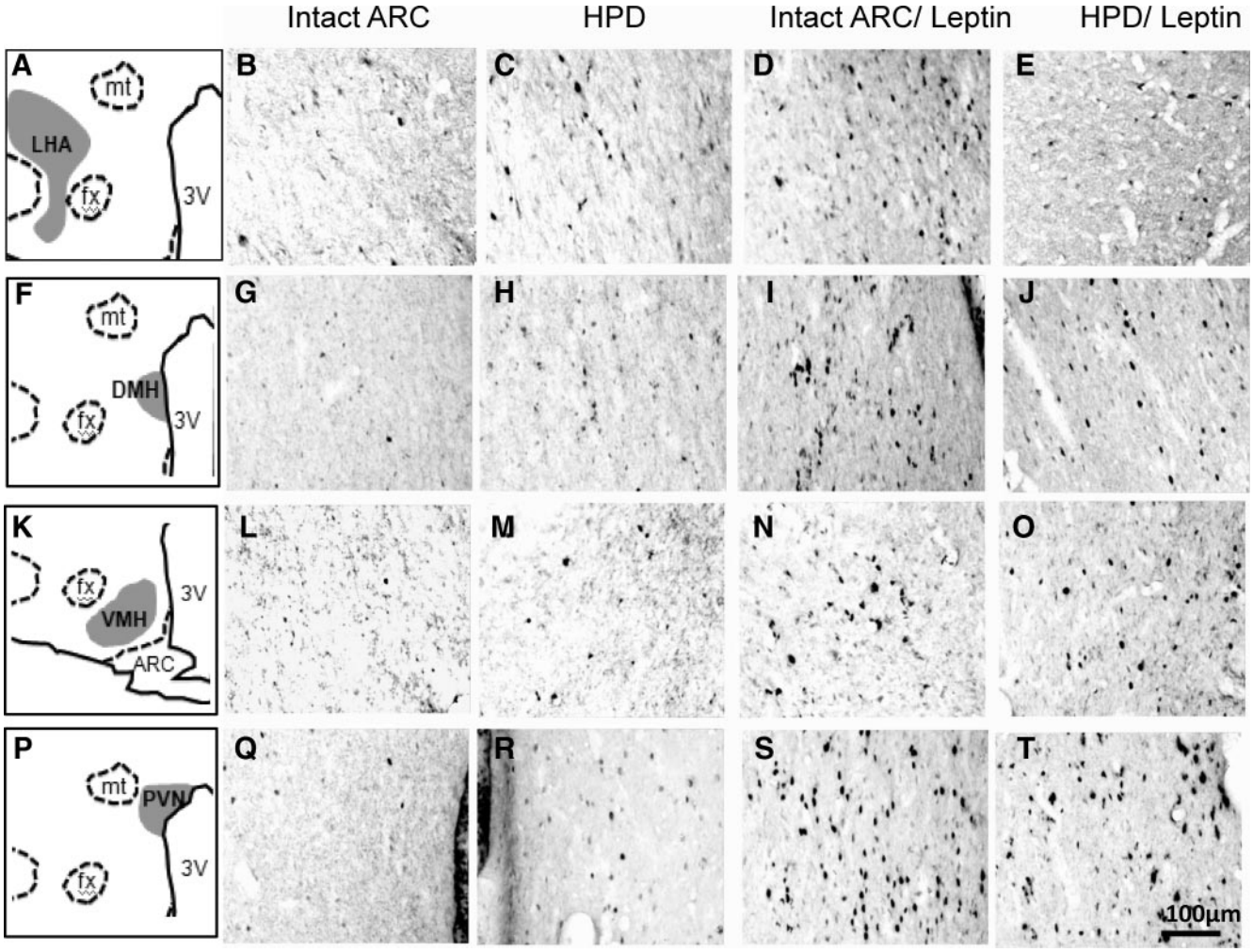

FIG. 5. Examples of Fos-immunostained neurons in different nuclei/areas of the ovine hypothalamus after leptin or vehicle treatment in ARC-intact and HPD animals. Fos-positive neurons are shown within the LHA (A–E), DMH (F–J), VMN (K–O), and the PVN (P–T). fx, Fornix; mt, mammillothalamic tract; 3V, third ventricle.

can act directly at the PVN, VMH, and DMH. Thus, convincing evidence has been obtained to show that cells in various regions of the hypothalamus respond to leptin in the absence of the ARC, supporting earlier studies that show that the first-order/second-order model of leptin signaling requiring a primacy of action in the ARC is not an exclusive mechanism for leptin action on appetite-regulating centers.

There are several approaches that can be employed to eliminate the ARC. Given its small size and mediobasal position closely adjacent to the third ventricle, bilateral lesions that are restricted to the nucleus have not been widely successful in rodents. The HPD operation in sheep, which involves complete destruction of the ARC bilaterally, causes animals to gain weight through increased adiposity and dysregulation of the seasonal control of food intake (31). On the other hand, the use of the neurotoxin, monosodium glutamate (MSG) to destroy the ARC yields variable results with respect to body weight. For example, it has been shown that after the partial destruction of the ARC by MSG treatment, animals display reduced weight gain, body fat mass, and plasma leptin levels (38). This is interpreted as arising from a sparing of the anorexigenic POMC neurons and a subsequent increase in energy expenditure without reduction in energy intake (39). This result is not universal, however, and in fact, most studies employing MSG lesions of the ARC result in a tendency toward obesity with a lesion that involves both orexigenic and anorexigenic neurons (40). Nonetheless, the ability of leptin to induce satiety is attenuated in MSG-treated rats (41). In both models, the importance of the ARC is demonstrated, but both orexigenic and anorectic neurons of this region are removed in our ARC ablation model.

The HPD operation that we used in this study is such that the hypothalamo-neurohypophysial tract is sectioned and the posterior lobe of the pituitary gland atrophies (30). The HPD operation totally sections all neurons projecting to the posterior lobe, including the oxytocin projections and this could be a cause of a reduction in oxytocin cell numbers in the hypothalamus in the HPD

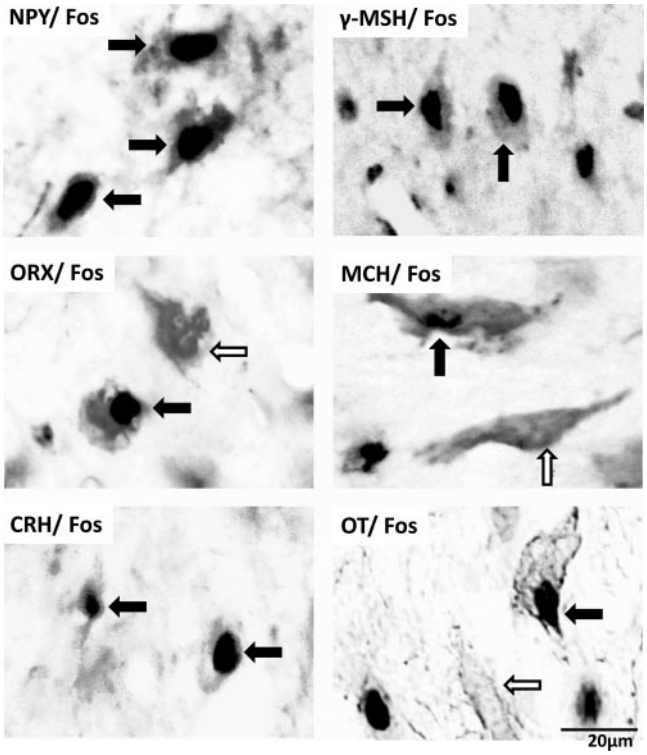

FIG. 6. Colocalization of various neuropeptides (diffuse cytoplasmic labeling indicated by either *black* or *white arrows*) and Fos immunostaining (dense nuclear label indicated by *black arrows*) after leptin administration. The *black arrows* indicate double-labeled neurons, and the *white arrows* represent neuropeptide staining in cells without Fos. ORX, Orexin; OT, oxytocin.

animals. This effect of the operation does not invalidate our results, because we were able to compare intact and HPD animals treated with either vehicle or leptin, and we confined our counting procedures to the region of the PVN where parvocellular neurons are found.

In general terms, there were discrepancies between the number of cells within a particular region that showed a fos response to leptin when cells of a specific type (in the same region) showed an opposite response. For example, in animals with an intact ARC, leptin caused a consistent increase in the number of fos-positive cells, whereas the number of NPY cells that were fos positive was significantly lowered by leptin. The obvious deduction from this is that cells other than those producing NPY (POMC and other) showed increased fos labeling. The identity of all cell types in the ARC that respond to leptin remains to be determined. Importantly, in the present study, we showed that the basal fos activity in NPY cells is reduced by leptin in the hypothalamo-pituitary intact animal, consistent with the anorexigenic action of leptin.

Our primary objective was to ascertain whether leptin may cause activation of hypothalamic cells in the absence of the ARC. Whereas this could be due to direct action on these cells, the possibility also exists that cells of the brainstem that project to the relevant cells of the hypothalamus may respond to leptin and cause activation. In this regard, we have documented projections of brainstem cells to the PVN (16) and the lateral hypothalamus (36). Leptin-receptive cells have been identified in the brainstem of rodents (42, 43) but not in sheep, and to conduct a systematic study of how such cells might be involved, combined neural tracing, fos labeling, and identification of cell types would be required; this was beyond the scope of the present study, which was specifically designed to test the first-order/second-order hypothesis of leptin signaling in the hypothalamus.

TABLE 2. Percentage of NPY cells in the ARC that project to the lateral hypothalamus

Sheep no.	% NPY cells retrogradely labeled
100	2.3 (4/173)
14	1.2 (3/243)
308	2.8 (4/142)
318	0.8 (2/237)
326	1.1 (3/265)
Mean ± SEM	1.7 ± 0.4

The numerator in the *parentheses* is the number of NPY cells retrogradely labeled in the ARC when FluoroGold was injected into the lateral hypothalamus, and the denominator is the total number of the NPY cells observed in three sections. These data are taken from the animals described in elsewhere (36).

Leptin action at the LHA is relayed via the ARC

Ablation of the ARC impacted on cells of the LHA, such that increased Fos labeling was seen in HPD animals. This suggests that the ARC predominantly provides tonic inhibition to the LHA, probably via POMC neurons, which are known to project to the LHA in rats and humans (13, 14). Our neuronal tracing results, however, show that the projections from the ARC to the LHA are not substantial in the ovine brain, and any direct input appears to be provided by the NPY neurons. Further substantiation of the weak connection between the ARC and the LHA in this species is the reported low level of melanocortin receptors in the latter (44). It seems unlikely, therefore, that increased activity of cells in the LHA after HPD is due entirely to removal of melanocortin signaling. In sheep, the LHA receives moderate projection from the VMN, DMH, and PVN (Y. Qi and I.J. Clarke, unpublished observations). These nuclei receive input from the ARC and also respond directly to leptin challenge (15, 16, 36), which indicates that the ARC may influence the LHA via an intermediary nucleus. We conclude that the cells of the ARC do not substantially dictate the function of the LHA in the sheep in a direct manner. One possible explanation of the increased Fos labeling in the LHA after removal of the ARC is that the LHA provides a degree

of redundant function in terms of appetite regulation so that removal of the ARC results in increased activity in the LHA to maintain homeostatic control.

Leptin effects in the DMH, VMH, and PVN are not relayed through the ARC

Cells in the DMH, VMH, and PVN responded to leptin irrespective of the presence or absence of the ARC, strongly supporting the case for direct action of leptin in these regions. Our observations of response of cells in the DMH and VMH are consistent with radioisotope binding studies in rodents, wherein leptin has been shown to bind to cells in these regions (45). Reduced binding of radiolabeled leptin in the DMH and VMH is evident in animals that are susceptible to diet-induced obesity (45), thus demonstrating that direct leptin action at these regions is likely to be important in determining individual susceptibility to obesity. This notion has been consolidated, at least for the VMH, whereby gene mutation studies have demonstrated that disruption of SF-1 in cells that contain leptin receptors significantly impairs leptin effects on satiety and body weight (28). In contrast, there is little evidence in rodents to support a direct effect of leptin on cells in the PVN, and the effect of leptin at this level is thought to be relayed through the ARC (reviewed in Ref. 8). Furthermore, neuronal tracing studies in sheep have demonstrated that strong connections extend from the ARC to the PVN (16). It must be noted, however, that further characterization of the chemical phenotype of cells (*vide infra*) unmasks a direct relationship between the ARC and the PVN. In summary, convincing evidence has been obtained to show that cells in various regions of the hypothalamus respond to leptin in the absence of the ARC, suggesting that the first-order to second-order model of leptin signaling (reviewed in Refs. 8 and 9) is not an exclusive mechanism for leptin action on appetite-regulating centers.

Neuropeptidergic characterization of Fos-responsive neurons

Despite the evidence, discussed above, providing an alternative to the exclusivity of the ARC in the mediation of the effects of peripheral leptin, it is well accepted that leptin acting on specific cells in this nucleus plays a major role in regulation of appetite and energy expenditure. In the present study, NPY cells showed reduced activity and POMC cells showed increased activity, as indicated by Fos labeling. This is consistent with the orexigenic properties of the former and the anorectic properties of the latter as well as the satiety inducing effect of leptin (12, 46, 47) (reviewed in Refs. 8, 9, and 46). It has been shown previously that leptin increases Fos labeling in cells of the rat hypothalamus (48, 49). In the study of Elias *et al.* (48), Fos labeling in the ARC was localized to cells producing cocaine- and amphetamine-related transcript (CART) and neurotensin. Cocaine- and amphetamine-related transcript is colocalized in cells that produce the melanocortins in the rat brain (50), and as such, these results are consistent with our present findings in the ovine brain, showing increased Fos labeling in POMC cells. Previous work in rodents has demonstrated a lack of effect of leptin on Fos expression in NPY cells (14). Increased Fos labeling of neurons is commonly used to show activation of cells, but a reduction in basal levels of Fos in neurons is less commonly reported. In this regard, it is salient to note that Fos labeling in hypothalamic nuclei such as the suprachiasmatic nucleus and the PVN is reduced under some circumstances (51–53). A good example of this is the reduction in Fos labeling seen in the PVN with naltrexone treatment (54). The reduction in Fos labeling in NPY cells after iv leptin is also consistent with reduced NPY gene expression after leptin administration to sheep (4) as well as other species (48, 49, 55) (reviewed in Ref. 56). The results of the present paper reinforce the fact that leptin increases activity of POMC cells and reduces activity of NPY neurons (12, 57).

Removal of the ARC elicited opposing effects on orexin and MCH neurons, such that HPD sheep exhibited a reduced Fos labeling in the former and increased Fos labeling in the latter. Analysis of orexin cells in the DMH was consistent with the observations in the LHA. This effect was also seen after leptin treatment, whereby leptin reduced the percentage of orexin cells containing Fos in HPD and control animals, but increased the percentage of MCH cells containing Fos, albeit in ARC-intact animals only. Although both MCH and orexin are characterized as orexigenic peptides (58, 59), the effects of these peptides on other functions such as arousal, sleep/wake cycles, and energy expenditure are thought to be divergent. Studies in rodents have demonstrated that orexins increase energy expenditure (60), whereas MCH reduces energy expenditure (61, 62). In addition, electrophysiological studies have demonstrated increased activity of orexin cells during periods of arousal, but MCH cell activity is increased during sleep (63). Furthermore, we have previously demonstrated different effects of altered adiposity on expression of genes for these two peptides. In lean ewes, expression of MCH mRNA is increased (64), but orexin mRNA levels are similar to animals of normal body weight (65). Given the apparent divergence in function of the MCH and orexin cells, it is not all that surprising that we see different effects of HPD surgery and leptin treatment on Fos expression in these cell types.

An important point to note was that in ARC-intact animals, leptin treatment increased Fos expression in MCH cells. In rodents, leptin treatment reduces MCH mRNA expression, consistent with the effect of leptin to reduce food intake and increase energy expenditure (66). We have previously reported increased MCH mRNA levels in sheep made lean via chronic food restriction (64), and MCH mRNA levels were inversely correlated to adiposity in genetically fat and lean sheep (67). In the current study, however, leptin treatment activated MCH neurons, which is counterintuitive to the effects of leptin and MCH on food intake. Furthermore, the effect of leptin on Fos expression in MCH cells was abolished in HPD animals. This demonstrates that unlike orexin, where effects of leptin were not relayed through the ARC, effects on MCH cells may have been effected via this nucleus. In fasted rodents, the increase in MCH mRNA is not directly linked to reduced melanocortin signaling (68), and effects may be mediated via the NPY cells. Nonetheless, as noted above, in sheep, few projections have been found to extend from the ARC directly to the LHA (*vide supra*), and therefore, the specific neuronal connectivity linking the ARC to the MCH neurons in the LHA in sheep requires further clarification.

In regard to the action of leptin via POMC and NPY neurons of the ARC, we inferred that this might involve demonstrated projections to the PVN (69–72). We have shown that removal of the ARC alters the response to peripheral administration of leptin in both CRH and oxytocin cells. Leptin administration increased the percentage of CRH cells containing Fos in ARC-intact animals only, indicating that effects of leptin on the CRH system are relayed via the ARC. In contrast, ablation of the ARC unmasked an effect of leptin on oxytocin neurons, where leptin treatment increased the percentage of oxytocin cells containing Fos in the HPD group alone. Thus, the current data demonstrate that the neural connection between the ARC and the PVN provide excitatory input to the CRH neurons, but inhibitory inputs to the oxytocin cells.

Conclusion

Removal of the ARC *per se* had an effect on the function of cells in other hypothalamic nuclei that are involved in energy balance, indicating its fundamental importance in overall homeostasis. In addition, we have clearly demonstrated that removal of the ARC can affect the response to leptin in some hypothalamic cell types but not others. These data are consistent with the notion that the ARC has a primary place in the central response to leptin, but particularly emphasizes the additional view that overall hypothalamic response to leptin involves an integrated response including direct action at the level of various appetite-regulating centers.

Acknowledgments

We thank Mr. Bruce Doughton and Ms. Linda Morrish for animal care. We thank Dr T. Sakurai, Dr. W. Vale, Dr. K. Ho, Dr. A. Lawrence, and Dr. J. Oliver for antibodies.

Address all correspondence and requests for reprints to: Prof. Iain Clarke, Department of Physiology, Building 13F, Monash University, Clayton, Victoria 3800, Australia. E-mail: iain.clarke@med.monash.edu.au.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432
2. **Ahima RS, Kelly J, Elmquist JK, Flier JS** 1999 Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia. Endocrinology 140:4923–4931
3. **Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM** 1997 Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. Proc Natl Acad Sci USA 94:8878–8883
4. **Henry BA, Goding JW, Alexander WS, Tilbrook AJ, Canny BJ, Dunshea F, Rao A, Mansell A, Clarke IJ** 1999 Central administration of leptin to ovariectomized ewes inhibits food intake without affecting the secretion of hormones from the pituitary gland: evidence for a dissociation of effects on appetite and neuroendocrine function. Endocrinology 140:1175–1182
5. **Clarke IJ, Henry B, Iqbal J, Goding JW** 2001 Leptin and the regulation of food intake and the neuroendocrine axis in sheep. Clin Exp Pharmacol Physiol 28:106–107
6. **Henry BA, Dunshea FR, Gould M, Clarke IJ** 2008 Profiling postprandial thermogenesis in muscle and fat of sheep and the central effect of leptin administration. Endocrinology 149:2019–2026
7. **Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern, PA, Friedman, JM** 1995 Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1:1155–1161
8. **Elmquist JK, Elias CF, Saper CB** 1999 From lesions to leptin: hypothalamic control of food intake and body weight. Neuron 22: 221–232
9. **Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG** 2000 Central nervous system control of food intake. Nature 404:661–671
10. **Faouzi M, Leshan R, Björnholm M, Hennessey T, Jones J, Münzberg H** 2007 Differential accessibility of circulating leptin to individual hypothalamic sites. Endocrinology 148:5414–5423
11. **Peruzzo B, Pastor FE, Blázquez JL, Schöbitz K, Peláez B, Amat P, Rodríguez EM** 2000 A second look at the barriers of the medial basal hypothalamus. Exp Brain Res 132:10–26
12. **Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL, Cone RD, Low MJ** 2001 Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411:480–484
13. **Elias CF, Saper CB, Maratos-Flier E, Tritos NA, Lee C, Kelly J, Tatro JB, Hoffman GE, Ollmann MM, Barsh GS, Sakurai T, Yanagisawa M, Elmquist JK** 1998 Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. J Comp Neurol 402:442–459
14. **Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, Bjorbaek C, Flier JS, Saper CB, Elmquist JK** 1999 Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron 23:775–786

15. **Qi Y, Iqbal J, Oldfield BJ, Clarke IJ** 2008 Neural connectivity in the mediobasal hypothalamus of the sheep brain. Neuroendocrinology 87:91–112
16. **Qi Y, Namavar MR, Iqbal J, Oldfield BJ, Clarke IJ** 2009 Characterization of the projections to the hypothalamic paraventricular and periventricular nuclei in the female sheep brain, using retrograde tracing and immunohistochemistry. Neuroendocrinology 90:31–53
17. **Levin MC, Sawchenko PE, Howe PR, Bloom SR, Polak JM** 1987 Organization of galanin-immunoreactive inputs to the paraventricular nucleus with special reference to their relationship to catecholaminergic afferents. J Comp Neurol 261:562–582
18. **Mihály E, Fekete C, Tatro JB, Liposits Z, Stopa EG, Lechan RM** 2000 Hypophysiotropic thyrotropin-releasing hormone-synthesizing neurons in the human hypothalamus are innervated by neuropeptide Y, agouti-related protein, and α-melanocyte-stimulating hormone. J Clin Endocrinol Metab 85:2596–2603
19. **Huang XF, Yu Y, Li Y, Tim S, Deng C, Wang Q** 2008 Ventromedial hypothalamic NPY Y2 receptor in the maintenance of body weight in diet-induced obesity in mice. Neurochem Res 33:1881–1888
20. **Fu LY, van den Pol AN** 2008 Agouti-related peptide and MC3/4 receptor agonists both inhibit excitatory hypothalamic ventromedial nucleus neurons. J Neurosci 28:5433–5449
21. **Münzberg H, Flier JS, Bjørbaek C** 2004 Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 145:4880–4889
22. **Golden PL, Maccagnan TJ, Pardridge WM** 1997 Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. J Clin Invest 99:14–18
23. **Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P** 1996 Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Lett 387:113–116
24. **Elmquist JK, Bjørbaek C, Ahima RS, Flier JS, Saper CB** 1998 Distributions of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol 395:535–547
25. **Iqbal J, Pompolo S, Murakami T, Grouzmann E, Sakurai T, Meister B, Clarke IJ** 2001 Immunohistochemical characterization of localization of long-form leptin receptor (OB-Rb) in neurochemically defined cells in the ovine hypothalamus. Brain Res 920:55–64
26. **Jacob RJ, Dziura J, Medwick MB, Leone P, Caprio S, During M, Shulman GI, Sherwin RS** 1997 The effect of leptin is enhanced by microinjection into the ventromedial hypothalamus. Diabetes 46:150–152
27. **Ambati S, Duan J, Choi YH, Hartzell DL, Della-Fera MA, Baile CA** 2009 ICV vs. VMH injection of leptin: comparative effects on hypothalamic gene expression. Behav Brain Res 196:279–285
28. **Dhillon H, Zigman JM, Ye C, Lee CE, McGovern RA, Tang V, Kenny CD, Christiansen LM, White RD, Edelstein EA, Coppari R, Balthasar N, Cowley MA, Chua Jr S, Elmquist JK, Lowell BB** 2006 Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. Neuron 49:191–203
29. **Montanaro MS, Allen AM, Oldfield BJ** 2005 Structural and functional evidence supporting a role for leptin in central neural pathways influencing blood pressure in rats. Exp Physiol 90:689–696
30. **Clarke IJ, Cummins JT, de Kretser DM** 1983 Pituitary gland function after disconnection from direct hypothalamic influences in the sheep. Neuroendocrinology 36:376–384
31. **Lincoln GA, Rhind SM, Pompolo S, Clarke IJ** 2001 Hypothalamic control of photoperiod-induced cycles in food intake, body weight, and metabolic hormones in rams. Am J Physiol Regul Integr Comp Physiol 281:R76–R90
32. **Li Q, Goodchild AK, Seyedabadi M, Pilowsky PM** 2005 Pre-protachykinin A mRNA is colocalized with tyrosine hydroxylase-immunoreactivity in bulbospinal neurons. Neuroscience 136:205–216
33. **Mercer LD, Le VQ, Nunan J, Jones NM, Beart PM** 2000 Direct visualization of cholecystokinin subtype2 receptors in rat central nervous system using anti-peptide antibodies. Neurosci Lett 293:167–170
34. **Adam CL, Archer ZA, Findlay PA, Thomas L, Marie M** 2002 Hypothalamic gene expression in sheep for cocaine- and amphetamine-regulated transcript, pro-opiomelanocortin, neuropeptide Y, agouti-related peptide and leptin receptor and responses to negative energy balance. Neuroendocrinology 75:250–256
35. **Richard P** 1967 Atlas stéréotaxique du cerveau de brebis Pré-Alpes-du-Sud. Paris: Institut national de la recherche agronomique
36. **Qi Y, Oldfield BJ, Clarke IJ** 2009 Projections of RFamide-related peptide-3 neurons in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. J Neuroendocrinol 21:690–697
37. **Chaillou E, Baumont R, Tramu G, 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
38. **Meister B, Ceccatelli S, Hökfelt T, Andén NE, Andén M, Theodorsson E** 1989 Neurotransmitters, neuropeptides and binding sites in the rat mediobasal hypothalamus: effects of monosodium glutamate (MSG) lesions. Exp Brain Res 76:343–368
39. **Kondoh T, Torii K** 2008 MSG intake suppresses weight gain, fat deposition, and plasma leptin levels in male Sprague-Dawley rats. Physiol Behav 95:135–144
40. **Bergen HT, Mizuno TM, Taylor J, Mobbs CV** 1998 Hyperphagia and weight gain after gold-thioglucose: relation to hypothalamic neuropeptide Y and proopiomelanocortin. Endocrinology 139:4483–4488
41. **Dawson R, Pelleymounter MA, Millard WJ, Liu S, Eppler B** 1997 Attenuation of leptin-mediated effects by monosodium glutamate-induced arcuate nucleus damage. Am J Physiol 273:E202–E206
42. **Huo L, Maeng L, Bjorbaek C, Grill HJ** 2007 Leptin and the control of food intake: neurons in the nucleus of the solitary tract are activated by both gastric distension and leptin. Endocrinology 148:2189–2197
43. **Hosoi T, Kawagishi T, Okuma Y, Tanaka J, Nomura Y** 2002 Brain stem is a direct target for leptin's action in the central nervous system. Endocrinology 143:3498–3504
44. **Iqbal J, Pompolo S, Dumont LM, Wu CS, Mountjoy KG, Henry BA, Clarke IJ** 2001 Long-term alterations in body weight do not affect the expression of melanocortin receptor-3 and -4 mRNA in the ovine hypothalamus. Neuroscience 105:931–940
45. **Irani BG, Dunn-Meynell AA, Levin BE** 2007 Altered hypothalamic leptin, insulin, and melanocortin binding associated with moderate-fat diet and predisposition to obesity. Endocrinology 148:310–316
46. **Harvey J, Ashford ML** 2003 Leptin in the CNS: much more than a satiety signal. Neuropharmacology 44:845–854
47. **Breen TL, Conwell IM, Wardlaw SL** 2005 Effects of fasting, leptin, and insulin on AGRP and POMC peptide release in the hypothalamus. Brain Res 1032:141–148
48. **Elias CF, Kelly JF, Lee CE, Ahima RS, Drucker DJ, Saper CB, Elmquist JK** 2000 Chemical characterization of leptin-activated neurons in the rat brain. J Comp Neurol 423:261–281
49. **Morrison CD, Morton GJ, Niswender KD, Gelling RW, Schwartz MW** 2005 Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling. Am J Physiol Endocrinol Metab 289:E1051–E1057
50. **Larsen PJ, Seier V, Fink-Jensen A, Holst JJ, Warberg J, Vrang N** 2003 Cocaine- and amphetamine-regulated transcript is present in hypothalamic neuroendocrine neurones and is released to the hypothalamic-pituitary portal circuit. J Neuroendocrinol 15:219–226
51. **Girotti M, Weinberg MS, Spencer RL** 2009 Diurnal expression of functional and clock-related genes in the rat HPA axis: system-wide shifts in response to restricted feeding. Am J Physiol Endocrinol Metab 296:E888–E897
52. **Tran PV, Fretham SJ, Carlson ES, Georgieff MK** 2009 Long-term reduction of hippocampal BDNF activity following fetal-neonatal iron deficiency in adult rats. Pediatr Res 65:493–498
53. **Tanuri FC, de Lima E, Peres MF, Cabral FR, da Graça Naffah-**

Mazzacoratti M, Cavalheiro EA, Cipolla-Neto J, Zukerman E, Amado D 2009 Melatonin treatment decreases c-fos expression in a headache model induced by capsaicin. J Headache Pain 10:105–110
54. **Beckman TR, Shi Q, Levine AS, Billington CJ** 2009 Amygdalar opioids modulate hypothalamic melanocortin-induced anorexia. Physiol Behav 96:568–573
55. **Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK** 1998 Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron 21:1375–1385
56. **Elmquist JK** 2001 Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. Physiol Behav 74:703–708
57. **Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ** 2001 The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int J Obes Relat Metab Disord 25(Suppl 5):S63–S67
58. **Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, Maratos-Flier E** 1996 A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature 380:243–247
59. **Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M** 1998 Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:573–585
60. **Teske JA, Billington CJ, Kotz CM** 2008 Neuropeptidergic mediators of spontaneous physical activity and non-exercise activity thermogenesis. Neuroendocrinology 87:71–90
61. **Ito M, Gomori A, Ishihara A, Oda Z, Mashiko S, Matsushita H, Yumoto M, Ito M, Sano H, Tokita S, Moriya M, Iwaasa H, Kanatani A** 2003 Characterization of MCH-mediated obesity in mice. Am J Physiol Endocrinol Metab 284:E940–E945
62. **Astrand A, Bohlooly-Y M, Larsdotter S, Mahlapuu M, Andersén H, Tornell J, Ohlsson C, Snaith M, Morgan DG** 2004 Mice lacking melanin-concentrating hormone receptor 1 demonstrate increased heart rate associated with altered autonomic activity. Am J Physiol Regul Integr Comp Physiol 287:R749–R758
63. **Hassani OK, Lee MG, Jones BE** 2009 Melanin-concentrating hormone neurons discharge in a reciprocal manner to orexin neurons across the sleep-wake cycle. Proc Natl Acad Sci USA 106:2418–2422
64. **Henry BA, Tilbrook AJ, Dunshea FR, Rao A, Blache D, Martin GB, Clarke IJ** 2000 Long-term alterations in adiposity affect the expression of melanin-concentrating hormone and enkephalin but not proopiomelanocortin in the hypothalamus of ovariectomized ewes. Endocrinology 141:1506–1514
65. **Iqbal J, Henry BA, Pompolo S, Rao A, Clarke IJ** 2003 Long-term alteration in bodyweight and food restriction does not affect the gene expression of either preproorexin or prodynorphin in the sheep. Neuroscience 118:217–226
66. **Sahu A** 1998 Evidence suggesting that galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), proopiomelanocortin (POMC) and neuropeptide Y (NPY) are targets of leptin signaling in the hypothalamus. Endocrinology 139:795–798
67. **Anukulkitch C, Rao A, Pereira A, McEwan J, Clarke IJ** 25 November 2009 Expression of genes for appetite-regulating peptides in the hypothalamus of genetically selected lean and fat sheep. Neuroendocrinology 10.1159/000262283
68. **Morton GJ, Mystkowski P, Matsumoto AM, Schwartz MW** 2004 Increased hypothalamic melanin concentrating hormone gene expression during energy restriction involves a melanocortin-independent, estrogen-sensitive mechanism. Peptides 25:667–674
69. **Kennedy AR, Todd JF, Dhillo WS, Seal LJ, Ghatei MA, O'Toole CP, Jones M, Witty D, Winborne K, Riley G, Hervieu G, Wilson S, Bloom SR** 2003 Effect of direct injection of melanin-concentrating hormone into the paraventricular nucleus: further evidence for a stimulatory role in the adrenal axis via SLC-1. J Neuroendocrinol 15:268–272
70. **Kenney MJ, Weiss ML, Mendes T, Wang Y, Fels RJ** 2003 Role of paraventricular nucleus in regulation of sympathetic nerve frequency components. Am J Physiol Heart Circ Physiol 284:H1710–H1720
71. **Mueller PJ, Cunningham JT, Patel KP, Hasser EM** 2003 Proposed role of the paraventricular nucleus in cardiovascular deconditioning. Acta Physiol Scand 177:27–35
72. **Segar JL, Ellsbury DL, Smith OM** 2002 Inhibition of sympathetic responses at birth in sheep by lesion of the paraventricular nucleus. Am J Physiol Regul Integr Comp Physiol 283:R1395–R1403