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Short communication

Evidence for involvement of the melanocortin MC_4 receptor in the effects of leptin on food intake and body weight

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Abstract

The hypothesis that the melanocortin MC_4 receptor mediates the homeostatic effects of leptin was tested. Leptin (0.3 nmol, i.c.v.) lowered food intake at 4 and 24 h and body weight at 24 h. This effect was inhibited by pretreatment with an analogue of melanocyte stimulating hormone (MSH), the selective melanocortin MC_4 receptor antagonist HS014 (cyclic [AcCys^{11},D-Nal^{14},Cys^{18},Asp-NH$_2^{22}$]-β-$MSH_{11\text{-}22}$, 0.3 nmol, i.c.v.). HS014 alone at this dose did not modify food intake or body weight. At a higher dose (1.0 nmol, i.c.v.) HS014 stimulated food intake and this orexigenic effect of HS014 was attenuated by leptin pretreatment (0.3 nmol, i.c.v.). These results confirm earlier findings that leptin inhibits food intake and lowers body weight via the melanocortin system and suggest that leptin affects signalling at the melanocortin MC_4 receptor.

Keywords: Leptin; Melanocortin MC_4 receptor; HS014; Food intake; Hyperphagia; Body weight

1. Introduction

Leptin, a 167-amino-acid polypeptide secreted from adipose tissue, inhibits food intake in rodents (Auwerx and Staels, 1998). The inhibition of food intake by leptin is mediated by the long form of the leptin receptor, which is primarily expressed in the hypothalamus and in several sites in the hindbrain of mice and rats (Mercer et al., 1998). Although intravenous injections of leptin activate several cell groups in the hypothalamus (Elmquist et al., 1998), the potential downstream messengers of leptin's action in the brain have not been firmly identified. It has been proposed that several anorectic peptides, including corticotropin-releasing factor (CRF), neurotensin, galanin, neuropeptide Y and pro-opiomelanocortin (POMC)-derived peptides, mediate the homeostatic effects of leptin (Costa et al., 1997; Gardner et al., 1998; Raber et al., 1997; Seeley et al., 1997; Sahu, 1998; Satoh et al., 1998; Uehara et al., 1998).

Five melanocortin receptor subtypes (MC_1–MC_5) have been cloned and MC_1, MC_3 and MC_4 receptors have been found in the rat brain. There is considerable evidence that melanocortin receptors are involved in the regulation of feeding behaviour. Ectopic overexpression of agouti peptide, an antagonist at melanocortin MC_3/MC_4 receptors (Klebig et al., 1995; Kiefer et al., 1997; Ollmann et al., 1997), and targeted mutation of the melanocortin MC_4 receptor cause obesity in mice (Huszar et al., 1997). The non-selective melanocortin receptor antagonist SHU9119 has been shown to enhance nocturnal feeding and block the inhibitory effects of melanocortin receptor agonist on food intake (Fan et al., 1997). Moreover, selective melanocortin MC_4 receptor blockade has been shown to stimulate food intake in rats (Kask et al., 1998a,b). Recently, it was found that a non-selective melanocortin receptor antagonist SHU9119 antagonised the inhibitory effects of leptin on food intake and body weight in rats (Seeley et al., 1997; Satoh et al., 1998). These data suggest that the central effects of leptin may involve changes in signalling at one or several of the melanocortin receptor subtypes.

The development of obesity in melanocortin MC_4 receptor-deficient mice and the stimulation of feeding upon blockade of the melanocortin MC_4 receptor suggest that the melanocortin MC_4 receptor could be an important mediator of leptin's effects on food intake. To test this hypothesis, rats were pretreated with first truly selective

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melanocortin MC_4 receptor antagonist, HS014 (cyclic [AcCys^{11},D-Nal^{14},Cys^{18},Asp-NH$_2^{22}$]-β-MSH$_{11-22}$, Schiöth et al., 1998), and the ability of leptin to reduce food intake and body weight was then examined. Studies in genetically obese animals have shown that the effects of leptin and POMC are independent and additive (Boston et al., 1997). However, it is not known whether feeding induced by melanocortin antagonists in non-obese animals can be inhibited by leptin. If the effects of leptin are indeed mediated by the melanocortin MC_4 receptor, it might be anticipated that leptin will inhibit the response to an orexigenic dose of HS014. This possibility was explored in the second experiment, where the order of drug administration was reversed and HS014 was applied in a dose causing maximal stimulation of food intake.

2. Materials and methods

2.1. Animals and surgery

Male Wistar rats (National Laboratory Animal Center, Kuopio, Finland) weighing 330–360 g at the time of surgery were individually housed in hanging wire mesh cages (45 × 37 × 19 cm) under controlled light (lights on from 0800 to 2000 h) and temperature (20–22°C), with free access to tap water and food (diet R35, Lactamin, Sweden). Rats were anaesthesised by intraperitoneal injection of chloral hydrate solution (350 mg 10 ml^{-1} kg^{-1}) and fixed in a stereotaxic frame (Kopf model 900, David Kopf instruments, Tujunga, CA, USA). The skull was exposed and permanent 23-gauge cannulas aimed at the left lateral ventricle were implanted as previously described (Kask et al., 1998b). Rats were allowed to recover for 7 days.

2.2. Procedure and injections

On test days the procedure was identical in all experiments. Food was removed from the cage and each rat was injected. Drugs or vehicle was injected over 1 min in a volume of 5 μl, through a 33-gauge injector connected to a 50-μl Hamilton syringe and infusion pump (World Precision Instruments, Sarasota, CA, USA) with polyethylene tubing. The movement of an air bubble in the tubing confirmed drug flow. Following the injection, the needle was left in place for 15 s and the cannula was closed with a stylet. Immediately after the injection the rats were returned to home cage. The second injection was given 30 min later and then the rats were given preweighed amount of food pellets (20–25 g) on clean plastic Petri dishes. The remaining food together with any spillage collected from stainless steel plates placed beneath the cages, was weighted to the nearest 0.01 g, using a Mettler balance. The amount of ingested food was calculated by subtracting the remaining food and any spillage from the amount of food presented. All injections were carried out between 1400 h and 1530 h.

2.3. Experimental protocols

2.3.1. Experiment 1

The rats were pretreated with the selective melanocortin MC_4 receptor antagonist HS014 (0.3 nmol, i.c.v.) 30 min before i.c.v. injection of leptin (0.3 nmol). This dose of HS014, which alone causes an increase in food intake only at 1 h but does not have long-term effects, was selected on the basis of our previous study (Kask et al., 1998b). The effective dose of leptin was found in a pilot experiment. Food intake was determined at 4 and 24 h and body weight was measured after the first injection and at 24 h.

2.3.2. Experiment 2

In this experiment we examined whether pretreatment with leptin reduces the orexigenic effects of selective melanocortin MC_4 receptor blockade. The rats were injected i.c.v with (1) vehicle and vehicle; (2) vehicle and 1.0 nmol of HS014, a dose causing maximal stimulation of food intake (Kask et al., 1998b); (3) leptin (0.3 nmol) and HS014 (1.0 nmol); (4) leptin (0.06 nmol) and HS014 (1.0 nmol) or (5) leptin (0.3 nmol) and vehicle. The drugs were injected 30 min apart. The food intake was measured at 1, 2, 3 and 4 h after the last injection.

2.4. Verification of injection sites

At the end of the study, the rats were overdosed with chloral hydrate (600 mg kg^{-1}) and Fast Green solution was injected to mark the injection site. Brains were removed and inspected for the distribution of the dye. Only data from the animals with a uniform distribution of the dye in the ventricles were used in the data analysis.

2.5. Drugs

Recombinant mouse leptin, purchased from R&D Systems (Abingdon, UK), was dissolved in sterile 15 mM HCl and brought to about pH 5.2 with sterile 7.5 mM NaOH. HS014 (available from Neosystem, Strasbourg, France) was synthesised by using a solid phase approach and purified by HPLC (high-performance liquid chromatography) as described earlier (Schiöth et al., 1998). The correct molecular weight of the peptide was confirmed by mass spectrometry. HS014 was dissolved in sterile distilled water and stored in aliquots at −20°C. The final dilution was made with Ringer's fluid.

2.6. Statistics

The data from food intake tests were processed with the Statistica 5.0 for Windows program (Statsoft, Tulsa, OK, USA). All data are expressed as cumulative values and are

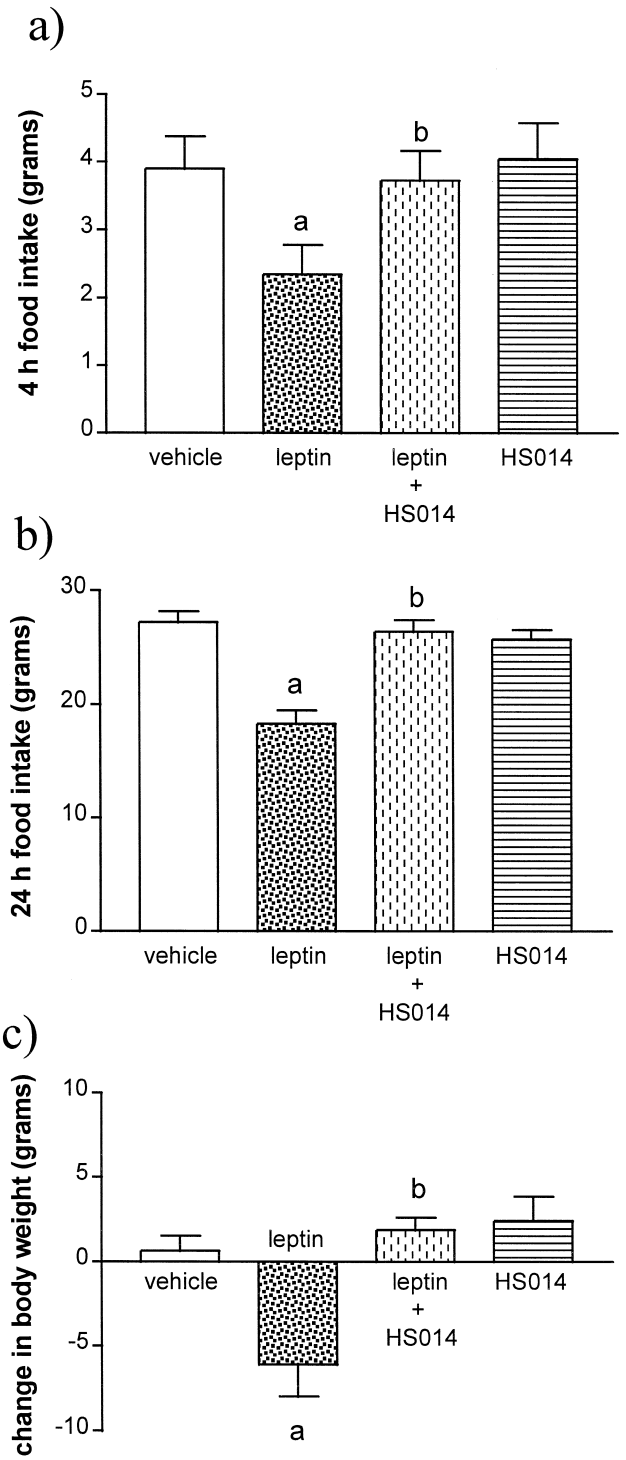

Fig. 1. Effect of pretreatment with HS014 (0.3 nmol) on the anorectic and weight-reducing effect of leptin (0.3 nmol). Data are expressed as means ± S.E.M. Rats were injected with: (1) vehicle and vehicle; (2) vehicle and leptin; (3) HS014 and leptin and (4) HS014 and vehicle. Food intake was measured at (a) 4 h and (b) 24 h and body weight (c) at 24 h after the last injection. [a]$P < 0.05$ vs. vehicle–vehicle group; [b]$P < 0.05$ vs. vehicle–leptin group (LSD test after significant ANOVA).

presented as means ± S.E.M.. Data were analysed by one-way analysis of variance (ANOVA) for factorial or repeated measures followed by the least significant difference (LSD) test where appropriate. Differences were considered to be significant when $P < 0.05$.

2.7. Animal ethics

Experimental procedures were approved by the Ethics Committee of Animal Experiments at the University of Tartu and were carried out in accordance with guidelines described in the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

3. Results

In the first experiment the rats were treated i.c.v. with the following drug combinations: (1) vehicle + vehicle; (2) vehicle + leptin (0.3 nmol); (3) HS014 (1 nmol) + leptin (0.3 nmol) and (4) HS014 (1 nmol) + vehicle. Factorial ANOVA indicated that these treatments had a significant effect on food intake at 4 h and 24 h ($F(3,34) = 2.94$ and 17.44 $P < 0.05$ and $P < 0.0001$, respectively). Post-hoc comparisons with the LSD test indicated that leptin alone caused a reduction in food intake at 4 h (50%, Fig. 1a) and at 24 h (33%, Fig. 1b). This effect was completely blocked by pretreatment with HS014 at 0.3 nmol, a dose which when given alone had no effect on food intake at 4 and 24 h. Leptin had a significant effect on body weight at 24 h ($F(3,34) = 6.3$, $P < 0.005$, Fig. 1c). The weight loss in leptin-treated rats was completely blocked by the pretreatment with 0.3 nmol of HS014. When given alone, this dose of HS014 did not significantly modify body weight at 24 h.

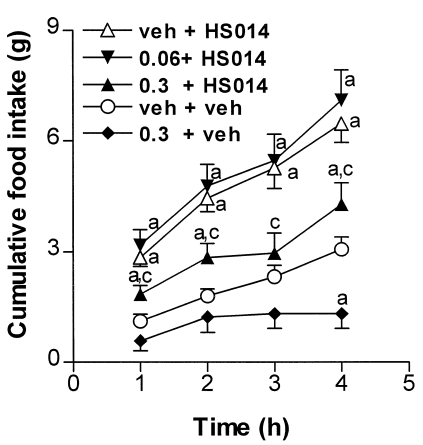

Fig. 2. The effect of pretreatment with leptin (0.06 and 0.3 nmol, only the dose of leptin is shown on inserted legend) on the orexigenic response to HS014 (1.0 nmol). Data are expressed as means ± S.E.M. Drugs or vehicle (veh) was injected into the lateral cerebral ventricle 30 min apart. [a]$P < 0.05$ vs. vehicle–vehicle group; [c]$P < 0.05$ vs. vehicle-HS014 group (LSD test after significant ANOVA).

The results of the second experiment where leptin (0.06 and 0.3 nmol, i.c.v.) was administered 30 min before HS014 (1 nmol, i.c.v.) are shown in Fig. 2. ANOVA for repeated measures indicated that these treatments had a significant effect on food intake over 4 h and there was a significant treatment-time interaction ($F(12,174) = 3.68$, $P < 0.0001$). Food intake differed at 1, 2, 3 and 4 h—$F(4,58) = 14.72$; 14.64; 11.69 and 14.90 respectively, $P < 0.0001$. Post-hoc LSD test indicated that HS014 increased food intake at all time points (Fig. 2). In HS014-treated rats pre-treated with leptin (0.06 and 0.3 nmol) food intake was still increased at all points, except at 3 h for rats pretreated with 0.3 nmol of leptin. Individual comparisons with HS014-treated rats indicated that pretreatment with 0.3 nmol leptin significantly reduced the effect of HS014 at all time points whereas a lower dose of leptin (0.06 nmol) was ineffective (Fig. 2). Although the rats treated with leptin (0.3 nmol) and vehicle consumed less food already after 1 h, this difference was not significant. Leptin alone (0.3 nmol) significantly lowered the food intake at 4 h (Fig. 2).

4. Discussion

Our data show that the effects of leptin on food intake and body weight were blocked completely by a low dose of a selective melanocortin MC_4 receptor antagonist, HS014. These findings are in line with previous data obtained for SHU9119, the non-selective melanocortin receptor antagonist (Seeley et al., 1997; Satoh et al., 1998), and support the hypothesis that the inhibitory effects of leptin on food intake and body weight are mediated through melanocortic neurones (Seeley et al., 1997), apparently via the melanocortin MC_4 receptor.

The complete blockade of the effects of leptin on food intake and body weight was somewhat unexpected, as it has been suggested that neurones containing glucagon-like peptide-1 (Goldstone et al., 1997) and CRF (Costa et al., 1997; Gardner et al., 1998; Uehara et al., 1998) are also potential targets for leptin. Boston et al. (1997) studied the weight gain and sensitivity to leptin in double mutant mice which lack leptin and overexpress agouti protein. These mice gain weight faster than mice with a single mutation but still respond to leptin with a decrease in food intake and body weight. This suggests that the melanocortin MC_4 receptor is not a sole mediator of leptin's effects. Interestingly, it has recently been proposed that a cocaine- and amphetamine-regulated transcript, which is absent in obese animals and which is stimulated by leptin, mediates the anorectic effect of this hormone secreted from adipose tissue (Kristensen et al., 1998). It remains to be established how these numerous putative downstream messengers of leptin relate to each other and whether they mediate differentially the effects of leptin on food intake and energy expenditure.

In the second experiment, leptin pretreatment attenuated the orexigenic effects of a high dose of HS014. Our data show, for the first time, that feeding induced by weakening of central melanocortin MC_4-ergic tone in non-obese animals can be inhibited by leptin pretreatment. The possibility of crosstalk between leptin and melanocortic receptors is strengthened by findings indicating that POMC neurones express leptin receptor mRNA (Cheung et al., 1997) and that leptin treatment increases POMC-gene expression in the hypothalamus of mice and rats (Schwartz et al., 1997). POMC can be processed into α-MSH and des-acetyl-α-MSH, which can then compete with HS014 at melanocortin MC_4 receptors. Our data, however, do not totally exclude the possibility that the melanocortin MC_3 receptor is involved in feeding because HS014 is a low-affinity antagonist for the melanocortin MC_3 receptor (Schiöth et al., 1998). The hypothesis that both melanocortin MC_3 and MC_4 receptors may affect food intake deserves attention, since melanocortin MC_3 receptors have been found in hypothalamic areas involved in the regulation of feeding behaviour (Roselli-Rehfuss et al., 1993), and central mechanisms modulating food intake are highly redundant. It has been also proposed that the neural pathways activated by melanocortic peptides and leptin are different (Boston et al., 1997). In this case, the net influence of these systems will determine whether the feeding response is initiated.

In conclusion, we have demonstrated that a selective melanocortin MC_4 receptor antagonist HS014 prevents the anorectic and weight-reducing effects of leptin in rats. Furthermore, the stimulation of food intake induced by weakening of central melanocortinergic tone with HS014 was attenuated by leptin. These findings suggest that leptin interacts with melanocortin MC_4 receptor signalling to inhibit food intake and add further support to the hypothesis that central melanocortin receptors play an important role in the regulation of ingestive behaviour.

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References

Auwerx, J., Staels, B., 1998. Leptin. Lancet 351, 737–742.

Boston, B.A., Blaydon, K.M., Varnerin, J., Cone, R.D., 1997. Independent and additive effects of central POMC and leptin pathways on murine obesity. Science 278, 1641–1644.

Cheung, C.C., Clifton, D.K., Steiner, R.A., 1997. Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology 138, 4489–4492.

Costa, A., Poma, A., Martignoni, E., Nappi, G., Ur, E., Grossman, A., 1997. Stimulation of corticotropin-releasing hormone release by the obese (ob) gene product, leptin, from hypothalamic explants. Neuroreport 8, 1131–1134.

Elmquist, J.K., Ahima, R., Elias, C.F., Flier, J.S., Saper, C.B., 1998. Leptin activates distinct projections from the dorsomedial and ventromedial hypothalamic nuclei. Proc. Natl. Acad. Sci. USA 95, 741–746.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J., Cone, R.D., 1997. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385, 165–168.

Gardner, J.D., Rothwell, N.J., Luheshi, G.N., 1998. Leptin affects food intake via CRF-receptor-mediated pathways. Nature Neurosci. 1, 103.

Goldstone, A.P., Mercer, J.G., Gunn, I., Moar, K.M., Edwards, C.M.B., Rossi, M., Howard, J.K., Rasheed, S., Turton, M.D., Small, C., Heath, M.M., O'Shea, D., Steere, J., Meeran, K., Ghatei, M.A., Hoggard, N., Bloom, S.R., 1997. Leptin interacts with glucagon-like peptide-1 neurons to reduce food intake and body weight in rodents. FEBS Lett. 415, 134–138.

Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P., Lee, F., 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88, 131–141.

Kask, A., Rägo, L., Korrovits, P., Wikberg, J.E.S., Schiöth, H.B., 1998a. Evidence that orexigenic effects of melanocortin 4 receptor antagonist HS014 are mediated by neuropeptide Y. Biochem. Biophys. Res. Commun. 248, 245–249.

Kask, A., Rägo, L., Mutulis, F., Pähkla, R., Wikberg, J.E.S., Schiöth, H.B., 1998b. Selective antagonist for the melanocortin 4 receptor (HS014) increases food intake in free-feeding rats. Biochem. Biophys. Res. Commun. 245, 90–93.

Kiefer, L.L., Ittoop, O.R., Bunce, K., Truesdale, A.T., Willard, D.H., Nichols, J.S., Blanchard, S.G., Mountjoy, K., Chen, W.J., Wilkinson, W.O., 1997. Mutations in the carboxyl terminus of the agouti protein decrease agouti inhibition of ligand binding to the melanocortin receptors. Biochemistry 36, 2084–2090.

Klebig, M.L., Wilkinson, J.E., Geisler, J.G., Woychik, R.P., 1995. Ectopic expression of the agouti gene in transgenic mice causes obesity, features of type II diabetes, and yellow fur. Proc. Natl. Acad. Sci. USA 92, 4728–4732.

Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., Larsen, P.J., Hastrup, S., 1998. Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393, 72–76.

Mercer, J.G., Moar, K.M., Hoggards, N., 1998. Localization of leptin receptor (Ob-R) messenger ribonucleic acid in rodent hindbrain. Endocrinology 139, 29–34.

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

Raber, J., Chen, S., Mucke, L., Feng, L., 1997. Corticotropin-releasing factor and adenocorticotropic hormone as potential central mediators of OB effects. J. Biol. Chem. 272, 15057–15060.

Roselli-Rehfuss, L., Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Low, M.J., Tatro, J.B., Entwistle, M.-L., Simerly, R.B., Cone, R.D., 1993. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc. Natl. Acad. Sci. USA 90, 8856–8860.

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.

Satoh, N., Ogawa, Y., Katsuura, G., Numata, Y., Masuzaki, H., Yoshimasa, Y., Nakao, K., 1998. Satiety effect and sympathetic activation of leptin are mediated by hypothalamic melanocortin system. Neurosci. Lett. 249, 107–110.

Schiöth, H.B., Mutulis, F., Muceniece, R., Prusis, P., Wikberg, J.E.S., 1998. Discovery of novel melanocortin 4 receptor selective MSH analogues. Br. J. Pharmacol. 124, 75–82.

Schwartz, M.W., Seeley, R.J., Woods, S.C., Weigle, D.S., Campfield, A.L., Burn, P., Baskin, D.G., 1997. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 46, 2119–2123.

Seeley, R.J., Yagaloff, K.A., Fisher, S.L., Burn, P., Thiele, T.E., van Dijk, G., Baskin, D.G., Schwartz, M.W., 1997. Melanocortin receptors in leptin effects. Nature 390, 349.

Uehara, Y., Shimizu, H., Ohtani, K., Sato, N., Mori, M., 1998. Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin. Diabetes 47, 890–893.