FEBS Letters 415 (1997) 134–138 FEBS 19251

Leptin interacts with glucagon-like peptide-1 neurons to reduce food intake and body weight in rodents

Anthony P. Goldstone[a], Julian G. Mercer[b], Irene Gunn[a], Kim M. Moar[b], C. Mark B. Edwards[a], Michela Rossi[a], Jane K. Howard[a], Shahnawaz Rasheed[a], Mandy D. Turton[a], Caroline Small[a], Melanie M. Heath[a], Donal O'Shea[a], Joanna Steere[a], Karim Meeran[a], Mohammed A. Ghatei[a], Nigel Hoggard[c], Stephen R. Bloom[a,*]

[a]*Department of Endocrinology and Metabolic Medicine, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK*
[b]*Molecular Neuroendocrinology Unit, Rowett Research Institute, Aberdeen AB21 9SB, UK*
[c]*Molecular Physiology Group, Rowett Research Institute, Aberdeen AB21 9SB, UK*

Received 13 August 1997

Abstract The adipose tissue hormone, leptin, and the neuropeptide glucagon-like peptide-1 (7–36) amide (GLP-1) both reduce food intake and body weight in rodents. Using dual in situ hybridization, long isoform leptin receptor (OB-Rb) was localized to GLP-1 neurons originating in the nucleus of the solitary tract. ICV injection of the specific GLP-1 receptor antagonist, exendin(9–39), at the onset of dark phase, did not affect feeding in saline pre-treated controls, but blocked the reduction in food intake and body weight of leptin pre-treated rats. These findings suggest that GLP-1 neurons are a potential target for leptin in its control of feeding.

Key words: Leptin; GLP-1; Exendin(9–39); In situ hybridization; Nucleus of the solitary tract; Feeding

1. Introduction

Leptin, secreted by adipose tissue, reduces food intake and body weight through interactions with central nervous system (CNS) pathways involved in energy balance, particularly in the hypothalamus [1–3]. The nature of these interactions has yet to be fully determined, but previous work has identified neuropeptide Y (NPY) neurons in the arcuate nucleus (ARC) as a target [3–6]. However, there is evidence that other CNS pathways regulating feeding may mediate the actions of leptin [3,7–11].

Glucagon-like peptide-1 (7–36) amide (GLP-1) is produced by tissue specific posttranslational processing of preproglucagon in the CNS [12]. In the rodent, preproglucagon mRNA and GLP-1-like immunoreactivity (GLP-1-IR) are found in nucleus of the solitary tract (NTS) neuronal cell bodies in the brainstem, with projections to the paraventricular nucleus (PVN) [13]. GLP-1-IR and a single specific GLP-1 receptor have also been found throughout the CNS, particularly the hypothalamus and other areas involved in the control of feeding [13–16]. In rodents, acute intracerebroventricular (ICV) injection of GLP-1 reduces food intake and chronic administration reduces body weight [7,14–18]. Importantly, ICV injection of exendin(9–39), a specific GLP-1 receptor antagonist [14,19], increases food intake in several animal models [14,20], indicating that endogenous GLP-1 is an inhibitor of feeding.

We have therefore investigated the hypothesis that leptin interacts with GLP-1 neurons in the control of food intake. Using a probe specific for the long, signalling variant of the leptin receptor (OB-Rb) [2], co-localization of OB-Rb and preproglucagon mRNA was examined in the rodent brainstem by dual in situ hybridization. The effects of CNS GLP-1 receptor blockade, using ICV exendin(9–39), on the anorectic and weight reducing actions of leptin were then investigated.

2. Materials and methods

2.1. PCR and probe synthesis

An 89 bp probe to the GLP-1 coding region of the preproglucagon sequence was generated from mouse brainstem cDNA using specific polymerase chain reaction (PCR) primers (5′-GACATGCTGAAGGGACCTTTAC-3′; +290–+311 and 5′-GGCCTTTCACCAGCCAC-3′; +378–+362; GenBank Z46845). Reverse transcription using the Superscript Preamplification system (Gibco BRL) was performed on 1 μg of total RNA extracted using the Microfast Track kit (Invitrogen). PCR was performed on a Hybaid Touchdown thermal cycler using the following conditions: [94°C (4 min), 1 cycle]; [94°C (1 min), 48°C (1 min), 72°C (1 min), 35 cycles]; [72°C (10 min), 1 cycle]. The PCR reaction was as described previously [2]. Agarose gel electrophoresis confirmed the presence of a band of the expected size, which was purified using Wizard PCR preps (Promega) and cloned directly into pGEM-T (Promega). The sequence and orientation of the insert was confirmed by automated sequencing. Plasmids were linearized with *Sac*I or *Apa*I for transcription with T7 or SP6 RNA polymerase to generate antisense and sense riboprobes. The probe specific to the long intracellular domain of the leptin receptor (OB-Rb), a 533 bp product, was generated as described previously [2].

2.2. In situ hybridization

Lean 'Aston' mice were drawn from a colony maintained at the Rowett Research Institute. Animals were killed by cervical dislocation and brains removed rapidly and frozen on dry ice. The study of OB-Rb and GLP-1 mRNA co-expression in the brainstem of the mouse used techniques described in detail elsewhere [5]. 10 μm coronal hindbrain sections were simultaneously hybridized with ^{35}S-labelled OB-Rb (at concentrations of $1.5–2.5\times10^7$ cpm/ml) and digoxigenin-labelled GLP-1 riboprobes at 58°C. After hybridization, slides were treated as described previously [5]. Dried slides were coated with 3% collodion and autoradiographic emulsion (LM-1; Amersham). Brain-

*Corresponding author. Fax: (44) (181) 383 3142.
E-mail: sbloom@rpms.ac.uk

Abbreviations: ARC, arcuate nucleus; CC, central canal; c-FLI, c-Fos-like immunoreactivity; CNS, central nervous system; CRH, corticotrophin releasing hormone; GLP-1, glucagon-like peptide-1 (7–36) amide; GLP-1-IR, GLP-1-like immunoreactivity; ICV, intracerebroventricular; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; OB-Rb, long isoform of leptin receptor; PCR, polymerase chain reaction; PVN, paraventricular nucleus

PII S0014-5793(97)01103-4

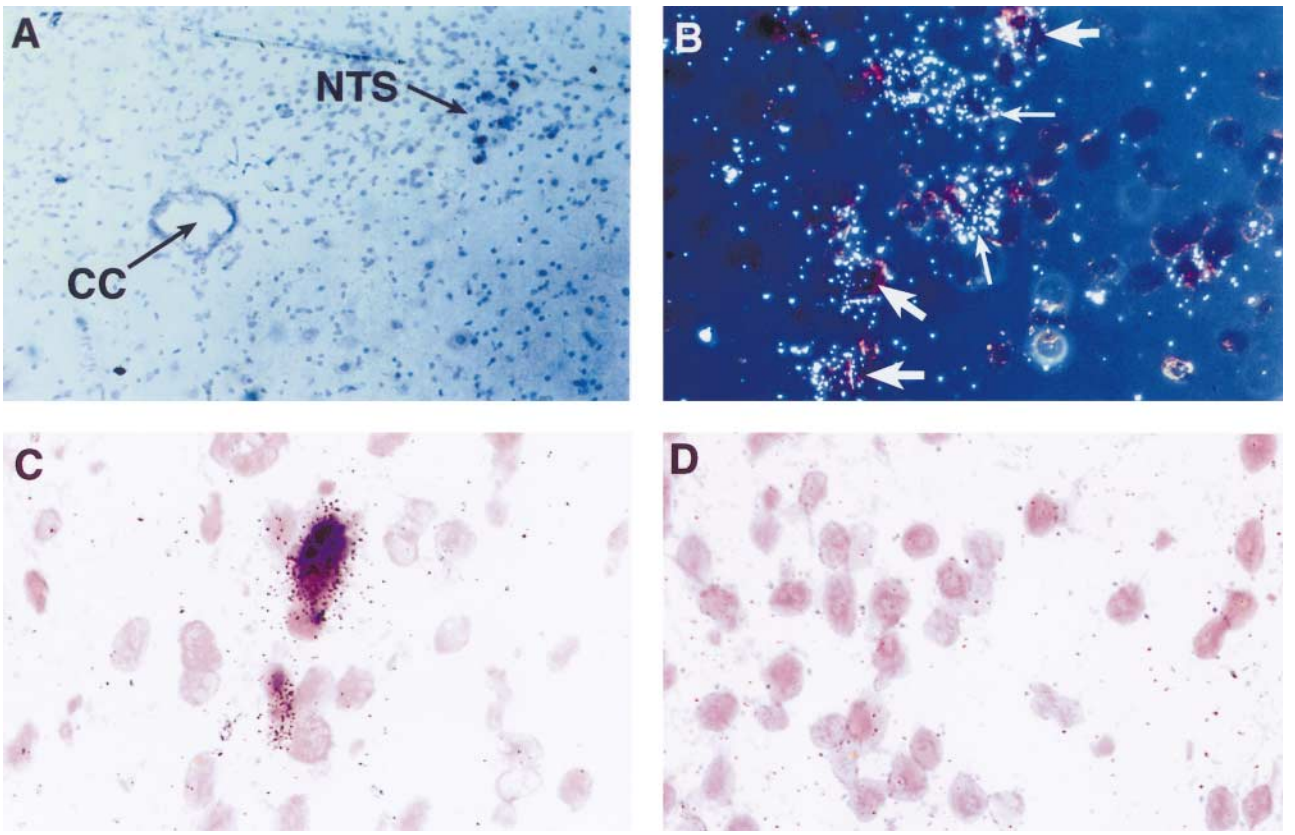

Fig. 1. In situ hybridization carried out on mouse hindbrain with ^{35}S-labelled leptin receptor (OB-Rb) and digoxigenin-labelled preproglucagon riboprobes. Photomicrographs of bright (A,C,D) or dark (B) field images of the NTS, generated following hybridization of adjacent sections with either both antisense probes (A,B,C) or both sense probes (D). The bright field image (A) reveals a cluster of NTS neurons that express preproglucagon mRNA (colored product). Silver grains (dark spots in bright field, white spots in dark field) representing OB-Rb mRNA expression are not visible at this magnification. At higher magnification, NTS neurons expressing both mRNA species are indicated by the association of silver grains with purple colored cells (B,C). Image B was obtained from the same cluster of NTS neurons shown in A, and shows cells that express both mRNA species (large arrows) as well as those that express OB-Rb but not preproglucagon mRNA (small arrows). Color formation or clustering of silver grains is not observed with sense probes (D). Magnification: A ×135; B,C,D ×650. Abbreviations: CC, central canal; NTS, nucleus of the solitary tract.

stem areas were identified by reference to adjacent stained sections and to atlases of the mouse and rat brain.

2.3. Peptides

Exendin(9–39) was synthesized using a peptide synthesizer and purified by reversed phase HPLC as previously described [14]. Recombinant mouse leptin was obtained from Novartis (Basel, Switzerland).

2.4. ICV cannulation and injection

Male Wistar rats (250–300 g), obtained from the colony kept at the Royal Postgraduate Medical School, were housed in individual cages under controlled temperature (21–23°C) and a 12 h light:dark cycle (lights on at 07.00 h), with free access to food (RM1 diet, SDS UK Ltd) and water. Animals were handled daily each morning at 09.00 h with measurement of body weight and food intake. Third ventricle cannulation and injection were performed as previously described [14,21]. Only animals with correct cannula placement, as confirmed by a sustained drinking response to ICV angiotensin II administration, were included. Compounds were dissolved in 0.9% saline and each 10 μl injection of peptide or saline was given over 1 min.

2.5. Effects of ICV leptin and exendin(9–39) on food intake and body weight

Four groups of animals were studied ($n = 28$–32 per group): (1) saline+saline, (2) saline+exendin(9–39), (3) leptin+saline and (4) leptin+exendin(9–39). Animals received an ICV injection of leptin (10 μg) or saline at 09.00 h on day 1. Animals were returned to their cages with food ad libitum, and injected ICV, 10 h later, at the onset of dark phase with either exendin(9–39) (100 μg) or saline. Food was weighed 2 and 4 h into the dark phase and subsequently each morning at 09.00 h for 4 days, when body weight was also measured. This dose of leptin produces a maximal effect on food intake [11], a finding confirmed by us in preliminary experiments. We have previously shown this dose of exendin(9–39) to increase feeding in the fed state [14].

2.6. Statistical analysis

Data are presented as means ± S.E.M. Differences between groups were initially analyzed using repeated measures ANOVA. When a significant group × time effect was found, individual time points were analyzed between groups by ANOVA with post hoc Tukey's test. Significance was taken as $P < 0.05$.

3. Results

3.1. In situ hybridization

Preliminary studies where alternate sections were hybridized with ^{35}S-labelled riboprobes to preproglucagon or OB-Rb mRNA suggested coexpression of these two genes in the rat NTS. However, the low levels of leptin receptor gene expression on emulsion-coated rat medullary sections required extended exposures (4–6 months). This resulted in poor resolution, preventing confident detection of mRNA co-localization. Dual in situ hybridization studies thus concentrated on murine tissue where leptin receptor mRNA was more abundant

(unpublished observations). Preproglucagon mRNA, as determined by formation of colored digoxigenin product (Fig. 1A), was restricted to a relatively small number of neurons in the caudal NTS. OB-Rb mRNA was expressed evenly across a wider area of the NTS. The majority, if not all, of the NTS neurons that expressed preproglucagon mRNA in sufficient quantity to generate a visible colored product also expressed the OB-Rb gene, as demonstrated by overlying silver grains (Fig. 1B,C). However, only 25–50% of neurons that expressed the receptor gene in the NTS also stained positive for preproglucagon mRNA. Colored product and silver grain clusters were not observed in the NTS following hybridization with the respective sense probes (Fig. 1D).

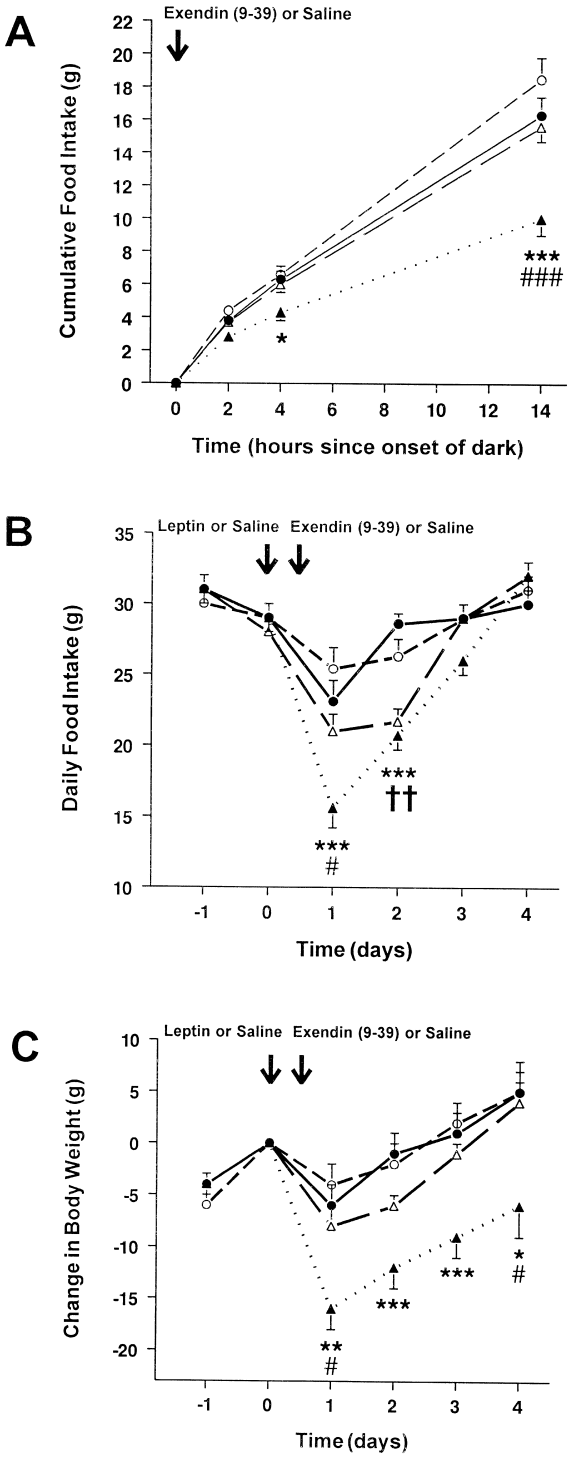

Fig. 2. Effects of leptin and exendin(9–39) on cumulative dark phase food intake on day 1 (A), total daily food intake (B) and daily body weight change relative to day 1 (C). Data presented as mean ± S.E.M. On day 1, leptin (10 μg) or saline was injected ICV, 10 h before onset of dark, followed by exendin(9–39) (100 μg) or saline ICV, at the onset of dark phase ($n = 28$–32 per group) [saline+saline: ●; saline+exendin(9–39): ○; leptin+saline: ▲; leptin + exendin(9–39): △]. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ leptin+saline vs. saline+saline; $P < 0.05$, $P < 0.005$ leptin+saline vs. leptin+exendin(9–39); $^{\dagger\dagger}P < 0.01$ leptin+exendin(9–39) vs. saline+exendin(9–39).

3.2. *Effects of ICV leptin and exendin(9–39) on food intake and body weight*

Light phase food intake on day 1 was reduced to a similar extent in both leptin pre-treated groups before the second injection (6.8 ± 0.5 g saline+saline, 6.9 ± 0.5 g saline+exendin(9–39), 5.6 ± 0.6 g leptin+saline, 5.4 ± 0.5 g leptin+exendin(9–39); $P < 0.01$ both leptin vs. both saline pre-treated groups). On day 1 no significant increase in dark phase food intake was seen after ICV exendin(9–39) administration in saline pre-treated animals at any time point (Fig. 2A). The total dark phase food intake of the leptin+saline group was reduced by 39 ± 6% on day 1 ($P < 0.001$ vs. saline+saline) (Fig. 2A). This was prevented by ICV exendin(9–39) administration (leptin+exendin(9–39): P = NS vs. saline+saline or saline+exendin(9–39), $P < 0.005$ vs. leptin+saline) (Fig. 2A). The percentage increase in total dark phase food intake after ICV exendin(9–39) administration was significantly greater in leptin pre-treated (56 ± 10%) than saline pre-treated animals (14 ± 8%, $P < 0.01$). The reversal of the anorectic effect of leptin by exendin(9–39) was apparent at 4 h (Fig. 2A), and continued over the rest of the dark phase. 24 h food intake on the second day, however (Fig. 2B), was similarly reduced by 28 ± 4% in the leptin+saline group ($P < 0.001$ vs. saline+saline) and by 24 ± 3% in the leptin+exendin(9–39) group ($P < 0.001$ vs. saline+saline, $P < 0.01$ vs. saline+exendin(9–39), P = NS vs. leptin+saline). By day 3 there was no significant difference in food intake between groups (Fig. 2B). Similarly, there was no significant effect of exendin(9–39) alone on body weight (Fig. 2C), but the reduction in body weight seen the day after ICV leptin injection ($P < 0.01$ vs. saline+saline) was prevented by ICV exendin(9–39) (leptin+exendin(9–39): P = NS vs. saline+saline or saline+exendin(9–39), $P < 0.05$ vs. leptin+saline).

4. Discussion

The CNS targets for the anorectic and weight reducing actions of leptin have yet to be fully identified, although previous work has suggested an action on hypothalamic NPY neurons. Leptin reduces ARC NPY gene expression and hypothalamic NPY release [3,4,6], [^{125}I]leptin uptake is seen in the ARC [22] and co-localization of NPY with leptin receptor gene expression can be demonstrated in ARC neurons [5]. However, leptin is still active in reducing feeding in the NPY knockout mouse and loss of NPY only partially reverses the effects of leptin deficiency in the *ob/ob*, NPY knockout cross [6,9]. ICV NPY is also less effective at increasing feeding in leptin treated rodents [10,11]. This suggests that leptin may interact with non-NPY expressing neurons in its control of food intake and body weight. Our work has previously dem-

onstrated that GLP-1 is an endogenous inhibitor of feeding [14], and we therefore tested the hypothesis that CNS GLP-1 neurons may be a target for leptin.

GLP-1 neuronal cell bodies were identified in the caudal NTS, a site of integration of many humoral and visceral inputs, as previously described [13]. In this study the demonstration by dual in situ hybridization of OB-Rb mRNA expression in almost all of the preproglucagon-expressing neurons in the NTS supports a possible interaction between leptin and CNS GLP-1, analogous to our previous findings with NPY neurons in the ARC [5]. The interaction may occur in the NTS itself, where c-Fos-like immunoreactivity (c-FLI) has been demonstrated following peripheral leptin administration in the rat [8]. Further co-expression studies will be required to confirm if these correspond to populations of NTS neurons expressing the GLP-1 and/or leptin receptor genes. Leptin might also act directly on nerve terminals to alter GLP-1 release from NTS projections to the PVN or the ARC, ventromedial and dorsomedial nuclei, or amygdala [13–15]. Both leptin and GLP-1 lead to neuronal activation in the PVN and amygdala, areas involved in feeding regulation [7,8,14]. In addition, some neuronal pathways are activated only by leptin, and others only by GLP-1 [7]. However this does not preclude their direct interaction since both peptides have multiple actions in the CNS [18,23,24]. The nature of the OB-Rb expressing neurons in the NTS that do not express preproglucagon also awaits further investigation.

The use of a specific GLP-1 antagonist is important if the functional effects of leptin on endogenous GLP-1 are to be examined. In this study no significant increase in feeding was seen in control animals given ICV exendin(9–39) at the onset of dark phase. Similar findings have been seen in the fasted state and, with a lower dose, over a 90 minute food restricted regime [14,18]. In contrast, ICV administration of exendin(9–39) at the beginning of the light phase after nocturnal feeding increases 2 h food intake [14]. The balance between endogenous GLP-1 activity and the onset and duration of action of ICV exendin(9–39) may explain these differences.

In contrast, ICV exendin(9–39) injection following ICV leptin administration produced a marked increase in total dark phase food intake, suggesting an increase in endogenous GLP-1 activity in these animals. The reduction in dark phase food intake and weight loss seen on the first day after ICV leptin administration was completely reversed by GLP-1 receptor blockade, implying a role for CNS GLP-1 in the anorectic and weight reducing effects of leptin. The fact that an increase in food intake and body weight with exendin(9–39) was only seen after leptin pre-treatment makes it unlikely that exendin(9–39) had a non-specific effect in its reversal of leptin action. On the second day, food intake was reduced to the same degree in the leptin+saline and leptin+exendin(9–39) animals, suggesting a normal response to the longer-acting ICV leptin after the GLP-1 receptor blockade with ICV exendin(9–39) had diminished.

The prolonged duration of GLP-1 receptor blockade following ICV exendin(9–39) administration implied by this study was confirmed in a separate experiment using exogenous GLP-1. Exendin(9–39) (100 μg) injected ICV 5 h before ICV GLP-1 (3 μg) administration at the onset of dark phase completely prevented the reduction in 2 h food intake seen with ICV GLP-1 alone [saline: 6.5 ± 0.4 g, GLP-1: 4.8 ± 0.5 g ($P < 0.05$ vs. saline), exendin(9–39): 6.9 ± 0.4 g ($P = $ NS), exendin(9–39)+GLP-1: 6.3 ± 0.4 g ($P = $ NS), unpublished observations].

Acute ICV GLP-1 administration reduces and acute ICV exendin(9–39) injection augments ICV NPY-induced feeding [14,25]. Chronic ICV exendin(9–39) increases NPY-induced weight gain [17]. It is therefore possible that the reduction in ICV NPY-induced feeding seen after ICV leptin in rodents is due to an increase in endogenous GLP-1 release [10,11]. Alternatively, leptin may have a direct effect on NPY-sensitive neurons or alter release of other neurotransmitters involved in feeding, such as corticotrophin releasing hormone (CRH) [3].

Although the GLP-1 receptor knockout mouse displays normal feeding behavior [16], it is noteworthy that this is also the case with the NPY knockout mouse [9]. Only with the leptin deficiency of the NPY −/−, *ob/ob* mouse cross does an effect on phenotype for the NPY knockout mouse become apparent [6]. The effect of leptin in the GLP-1 receptor knockout mouse and its cross with the *ob/ob* mouse will therefore be of interest. However, the numerous other CNS neuropeptides, in addition to NPY and GLP-1, through which leptin might act to control feeding (such as CRH, α-melanocyte stimulating hormone, melanin concentrating hormone and galanin circuits) suggests that redundancy and hierarchy are likely.

In conclusion, we have demonstrated expression of OB-Rb mRNA in GLP-1 neurons originating in the NTS. We have shown that the GLP-1 receptor antagonist, exendin(9–39), given ICV at the onset of the dark phase, increases dark phase feeding in leptin but not saline pre-treated animals, and prevents the anorectic and weight reducing effects of ICV leptin administration. This adds further support for a potential role of CNS GLP-1 in the control of feeding and body weight, and suggests that CNS GLP-1 neurons may be a target for the adipose hormone leptin. Further studies are needed to examine the nature of this interaction.

Acknowledgements: We wish to thank Michele Chiesi and Nigel Levens (Novartis, Basel, Switzerland) for providing recombinant mouse leptin; the UK Medical Research Council (A.P.G., M.M.H., J.K.H., K.M., S.R.B.), Scottish Office Agriculture, Environment and Fisheries Department (J.G.M., K.M.M., N.H.), Wellcome Trust (C.S.) for financial support; and David Morgan, Sang-Jeon Choi, Toshihiko Miyoshi, and David Sunter for their contributions to animal work.

References

[1] Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R. and Burn, P. (1995) Science 269, 546–549.
[2] Mercer, J.G., Hoggard, N., Williams, L.M., Lawrence, B., Hannah, L.T. and Trayhurn, P. (1996) FEBS Lett. 387, 113–116.
[3] Schwartz, M.W., Seeley, R.J., Campfield, L.A., Burn, P. and Baskin, D.G. (1996) J. Clin. Invest. 98, 1101–1106.
[4] Stephens, T.W., Basinski, M., Bristow, P.K., Bue-Valleskey, J.M., Burgett, S.J., Craft, L., Hale, J., Hoffmann, J., Hsiung, H.M., Kriauciunas, A., MacKellar, W., Rosteck, P.R., Schoner, B., Smith, D., Tinsley, F.C., Zhang, X.Y. and Heiman, M. (1995) Nature 377, 530–532.
[5] Mercer, J.G., Hoggard, N., Williams, L.M., Lawrence, C.B., Hannah, L.T., Morgan, P.J. and Trayhurn, P. (1996) J. Neuroendocrinol. 8, 733–735.
[6] Erickson, J.C., Hollopeter, G. and Palmiter, R.D. (1996) Science 274, 1704–1707.
[7] van Dijk, G., Thiele, T.E., Donahey, J.C.K., Campfield, L.A., Smith, F.J., Burn, P., Bernstein, I.L., Woods, S.C. and Seeley, R.J. (1996) Am. J. Physiol. 40, R1096–R1100.
[8] Elmquist, J.K., Ahima, R.S., Maratosflier, E., Flier, J.S. and Safer, C.B. (1997) Endocrinology 138, 839–842.

[9] Erickson, J.C., Clegg, K.E. and Palmiter, R.D. (1996) Nature 381, 415–418.
[10] Smith, F.J., Campfield, L.A., Moschera, J.A., Ballon, P.S. and Burn, P. (1996) Nature 382, 307.
[11] Stricker-Krongrad, A., Chiesi, M., Cumin, F., Spanka, C., Whitebread, S., Rentsch, J., Lollmann, B., Hofbauer, K.G. and Levens, N. (1997) Obesity Res. 4, 37s.
[12] Kreymann, B., Ghatei, M.A., Burnet, P., Williams, G., Kanse, S., Diani, A.R. and Bloom, S.R. (1989) Brain Res. 502, 325–331.
[13] Larsen, P.J., Tang-Christensen, M., Holst, J.J. and Orskov, C. (1997) Neuroscience 77, 257–270.
[14] Turton, M.D., O'Shea, D., Gunn, I., Beak, S.A., Edwards, C.M.B., Meeran, K., Choi, S.J., Taylor, G.M., Heath, M.M., Lambert, P.D., Wilding, J.P.H., Smith, D.M., Ghatei, M.A., Herbert, J. and Bloom, S.R. (1996) Nature 379, 69–72.
[15] Navarro, M., Rodriquez, D.E., Fonseca, F., Alvarez, E., Chowen, J.A., Zueco, J.A., Gomez, R., Eng, J. and Blazquez, E. (1996) J. Neurochem. 67, 1982–1991.
[16] Scrocchi, L.A., Brown, T.J., MaClusky, N., Brubaker, P.L., Auerbach, A.B., Joyner, A.L. and Drucker, D.J. (1996) Nat. Med. 2, 1254–1258.
[17] Turton, M.D., Edwards, C.M.B., Meeran, K., O'Shea, D., Gunn, I., Heath, M.M., Ghatei, M.A. and Bloom, S.R. (1997) Diabetes 46, (Suppl. 1) 185A.
[18] Tang-Christensen, M., Larsen, P.J., Goke, R., Finkjensen, A., Jessop, D.S., Moller, M. and Sheikh, S.P. (1996) Am. J. Physiol. 40, R848–R856.
[19] Goke, R., Fehmann, H.C., Linn, T., Schmidt, H., Krause, M., Eng, J. and Goke, B. (1993) J. Biol. Chem. 268, 19650–19655.
[20] Gunn, I., O'Shea, D., Turton, M.D., Beak, S.A. and Bloom, S.R. (1996) Biochem. Soc. Transac. 24, 581–584.
[21] Rossi, M., Choi, S.J., O'Shea, D., Miyoshi, T., Ghatei, M.A. and Bloom, S.R. (1997) Endocrinology 138, 351–355.
[22] Banks, W.A., Kastin, A.J., Huang, W., Jaspan, J.B. and Maness, L.M. (1996) Peptides 17, 305–311.
[23] O'Shea, D., Gunn, I., Chen, X., Bloom, S. and Herbert, J. (1996) NeuroReport 7, 830–832.
[24] Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E. and Flier, J.S. (1996) Nature 382, 250–252.
[25] Meeran, K., O'Shea, D., Turton, M.D., Heath, M.M., Gunn, I., Choi, S.J., Edwards, C.M.B., Small, C., Ghatei, M.A. and Bloom, S.R. (1996) Diabetes 45, (Suppl. 2) 170A.