Zurich Open Repository and Archive
University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2009

Blunted fasting-induced hypothalamic activation and refeeding hyperphagia in late-onset obesity

Becskei, C ; Lutz, Thomas A ; Riediger, Thomas

DOI: https://doi.org/10.1159/000251723

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-23381
Journal Article
Published Version

Originally published at:
Becskei, C; Lutz, Thomas A; Riediger, Thomas (2009). Blunted fasting-induced hypothalamic activation and refeeding hyperphagia in late-onset obesity. Neuroendocrinology, 90(4):371-382.
DOI: https://doi.org/10.1159/000251723

Appetite, Energy Balance and Obesity

Neuroendocrinology

Neuroendocrinology 2009;90:371–382
DOI: 10.1159/000251723

Received: February 5, 2009
Accepted: May 1, 2009
Published online: October 21, 2009

Blunted Fasting-Induced Hypothalamic Activation and Refeeding Hyperphagia in Late-Onset Obesity

Csilla Becskei Thomas A. Lutz Thomas Riediger

Institute of Veterinary Physiology and Zurich Centre of Integrative Human Physiology, University of Zurich, Zurich, Switzerland

Key Words
Arcuate nucleus, hypothalamic · Paraventricular nucleus, hypothalamic · Hypothalamic area, lateral · c-Fos · Obesity, late-onset

Abstract
Hormonal and metabolic factors signal the status of energy balance to hypothalamic nuclei. Obesity is characterized by neuronal, metabolic and hormonal alterations. We therefore hypothesized that hypothalamic responses to challenges of energy balance may differ between lean and obese animals. To test this, we compared c-Fos expression in the hypothalamic arcuate (ARC) and paraventricular nuclei (PVN) and the lateral hypothalamic area (LHA) of mice (1-year-old) with late-onset obesity (LOO) and of lean controls under different feeding conditions. Fourteen hours of fasting induced high c-Fos expression in neuropeptide-Y-positive ARC neurons, in the PVN and in the rostral LHA in lean but not in LOO mice. c-Fos expression in melanin-concentrating hormone (MCH) and orexin-containing neurons in the caudal LHA was not affected by fasting. LOO mice showed fasting hyperinsulinemia, hyperleptinemia, elevated fasting blood glucose and an attenuated hyperphagic response during refeeding. Moreover, the anorectic response to leptin and hypoglycemic response to insulin were reduced in LOO mice. We conclude that adiposity blunts the neuronal responses to metabolic challenges in hypothalamic centers which control feeding behavior and energy balance. Elevated blood glucose may be one factor that suppresses hypothalamic responsiveness in obese mice. A similar impact of hyperinsulinemia and hyperleptinemia in LOO mice is also likely although under the current experimental conditions responsiveness to some effects of these hormones appeared to be reduced.

Copyright © 2009 S. Karger AG, Basel

Introduction

Neurons expressing orexigenic and anorectic neuropeptides in the arcuate nucleus (ARC), the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) constitute a feeding regulatory circuit [1]. These include the orexigenic neuropeptide Y (NPY) and the anorectic proopiomelanocortin (POMC) neurons located in the ARC and the orexin and melanin-concentrating hormone (MCH) expressing neurons located almost exclusively in the caudal LHA. Neuronal activity correlates with the energy status in lean mice in all three nuclei [2–6]. Food deprivation increases c-Fos expression, a marker for neuronal activation, in these structures [2–6]. When energy balance is restored by chow refeeding c-Fos expression is rapidly reversed, supporting a crucial role

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
0028–3835/09/0904–0371$26.00/0

Accessible online at:
www.karger.com/nen

Thomas Riediger
Institute of Veterinary Physiology, University of Zurich
Winterthurerstrasse 260
CH–8057 Zurich (Switzerland)
Tel. +41 44 635 8815, Fax +41 44 635 8932, E-Mail triedig@vetphys.uzh.ch

of these brain sites in the maintenance of energy homeostasis [3–5]. Peripheral signals that may attune neuronal activity to the energy status include insulin, leptin, ghrelin and possibly metabolites such as glucose [1, 7–9].

Obesity is characterized by hyperleptinemia and leptin resistance and is often accompanied by several hormonal and metabolic alterations that are key symptoms of the so-called metabolic syndrome, including insulin resistance, hyperinsulinemia, hyperglycemia and hyperlipidemia [10–12]. Furthermore, changes in hypothalamic function in obesity are indicated by studies showing lower signal transducer and activator of transcription 3 phosphorylation in response to leptin in the ARC [13], reduced leptin-modulated NPY, agouti-related peptide and α-melanin-stimulating hormone release from ARC neurons [14], and reduced c-Fos response to glucose in the PVN and ARC [15]. Therefore, we hypothesized that the hypothalamic responsiveness to metabolic challenges differs between obese and lean individuals. To test this, we compared c-Fos expression in obese and lean mice under ad libitum feeding conditions and after 14 h of food deprivation. We also investigated whether the activated neurons express the orexigenic NPY in the ARC or orexin and MCH in the LHA.

We used mice that become obese at the age of 1 year while feeding on standard chow (late-onset obesity; LOO). This model resembles the most common form of human obesity in that it is characterized by a slow rate of fat accumulation over the course of years until early senescence. Obesity in humans and animals is generally characterized by hyperleptinemia and hyperinsulinemia and by reduced sensitivity to these hormones [10–12]. However, it is not known whether these are also characteristics of the LOO model. Therefore in the current study we also investigated leptin and insulin sensitivity in this model. Furthermore, this mouse model is not biased by genetic or dietary manipulation. To dissociate the effects of age and obesity in the LOO model, we also studied c-Fos expression in the hypothalamus of fasted lean mice of different ages (3–18 months).

To investigate whether the differences in the fasting-induced changes in hypothalamic activity between obese and lean mice might be associated with plasma insulin, leptin, ghrelin and metabolite levels we compared these parameters between lean and obese mice under the different feeding conditions. Energy expenditure and respiratory quotient were also determined as key indicators of energy homeostasis and substrate utilization. In order to test whether fasting-induced activation in the hypothalamic nuclei translates into a compensatory increase in food intake at the time of refeeding, we compared the feeding responses to food deprivation between lean and obese mice.

Methods

Animals

Adult male wild-type C57Bl/6 (Charles Rivers, Erkrath, Germany) and transgenic mice expressing green fluorescent protein (GFP) in POMC neurons (kind gift of B.B. Lowell, Beth Israel Deaconess Medical Center, Boston, Mass., USA) were housed individually in a temperature-controlled room (22°C) under a 12-hour light/dark cycle (lights off at 09.00 h). Animals were handled daily for at least 3 weeks before the experiments according to the procedure recommended by Ryabinin et al. [16] to reduce handling-induced c-Fos expression. The animals had free access to rodent chow (No. 3430; Kliba Nafag, Kaiseraugst, Switzerland), except when food-deprived (see below) for 14 h until 2 h after the onset of the dark phase (11.00 a.m.).

Chow-fed C57Bl/6 mice gained weight at a different rate. At the age of 12 months 30% of the mice weighed more than 39 g and were therefore defined as late-onset obese (LOO). Approximately 20% of the mice had an average body weight of 34 g at the age of 1 year and hence were considered lean. All animal procedures were approved by the Veterinary Office of the Canton of Zurich. All applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

Experiments

The effect of obesity on the fasting-induced hypothalamic c-Fos expression was investigated in 14-hour fasted 3-month-old lean (n = 11) and 1-year-old LOO mice (n = 6). Pertinent controls were fed ad libitum (n = 9 for lean and n = 6 for LOO). Lean mice weighed 27.7 ± 0.4 g and LOO mice weighed 45.0 ± 0.8 g.

In order to dissociate the effects of age and obesity, we investigated the fasting-induced c-Fos expression in 14-hour fasted lean mice across different age groups: at ages of 3 (n = 6; BW 25.7 ± 0.3 g), 9 (n = 6; BW 31.0 ± 0.6 g), 12 (n = 5; BW 33.0 ± 0.4 g) and 18 (n = 6; BW 33.5 ± 0.6 g) months. At the time of sacrifice, the perirenal and epididymal fat pads were removed and weighed.

The phenotype of the ARC neurons activated after fasting was analyzed in lean young POMC-GFP mice (n = 3, BW 29.5 ± 1.0 g). In the hypothalamic sections of these animals c-Fos immunohistochemistry was coupled with in situ hybridization histochemistry for NPY mRNA and with immunohistochemistry for GFP (see below).

The effect of fasting on compensatory hyperphagia was investigated in LOO (n = 8) and 3-month-old lean (n = 8) mice. Baseline food intake was measured for 3 days in ad libitum-fed lean and LOO mice by a computerized system. Then all animals were fasted for 12 h during the light phase. Following refeeding at dark onset, cumulative food intake was analyzed at 1 and 12 h and was compared to the mean of the baseline values measured at the same time intervals in ad libitum-fed animals. In pilot studies 12-month-old lean animals showed a hyperphagic response to 12 h of food deprivation. 12-Hour cumulative food intake in ad libi-

tum-fed and fasted animals was 3.1 ± 0.1 and 3.9 ± 0.1 g, respectively ($p = 0.003$). Because based upon these results age did not seem to have a major effect on fasting-induced hyperphagia in our model, we used young lean mice as controls in the current study.

The anorectic effect of leptin was tested in ad libitum-fed LOO (n = 6; BW 40.8 ± 0.8 g) and age-matched lean mice (n = 6; BW 33.7 ± 0.7 g). In these experiments age-matched lean controls were used because 12-month-old lean mice had much higher leptin levels than the 3-month-old lean counterparts and because leptin levels are believed to influence leptin sensitivity [17]. In the current experiment animals received a subcutaneous (s.c.) injection of saline first (0.1 ml/10 g) and 3 days later leptin (2.6 mg/kg in saline, 0.1 ml/10 g) at dark onset. Following injection mice were given access to chow and food intake was measured manually 2, 4, 6, 8, 10, 12 and 24 h later. Food intake was corrected for spillage. For each animal the difference in food intake compared to saline injection was calculated.

The effect of insulin on blood glucose levels was investigated in 3-month-old lean (n = 6; BW 31.2 ± 0.6 g) and LOO mice (n = 9; BW 44.5 ± 1.6 g). We used young lean mice in this experiment because there was no indication of an age-related effect on insulin sensitivity at the age of 12 months in our model. In the immunohistological experiments the serum insulin and blood glucose level of the 12-month-old lean mice did not show any difference compared to the 3-month-old lean counterparts. This was in contrast to the large differences in fasting insulin and glucose values between 12-month-old LOO mice and their young lean controls. In the current experiment animals were food deprived for 12 h during the light phase. At dark onset a drop of blood was obtained by tail tip amputation from unanesthetized mice and was analyzed by a portable glucometer (Glucometer Elite, Bayer, Leverkusen, Germany). Then the animals were injected with insulin (0.1 U/kg s.c.) or an equivalent volume (0.1 ml/10 g) of saline, and were returned to their home cages. Blood sampling was repeated 10, 20, 30, 45 and 60 min later. Changes in blood glucose levels compared to the baseline value were calculated for each animal and used for statistical analysis.

The effect of fasting on metabolic parameters was studied in LOO (n = 6; BW 40.1 ± 0.7 g) and age-matched lean mice (n = 6; BW 32.2 ± 0.3 g) using an indirect calorimetry system. Energy expenditure, respiratory quotient and locomotor activity were determined in an open circuit system (AccuScan Inc., Columbus, Ohio, USA) as reported previously [18]. The energy expenditure over 12 h was calculated for each animal and was approximated for the metabolic body weight (body weight$^{0.75}$). The fasting-induced changes (in %) in energy expenditure compared to ad libitum-fed values were calculated for all animals and used for statistical analysis.

Immunohistochemistry

Animals were euthanized 2 h into the dark phase and were processed as reported previously [4–6]. Three separate series of hypothalamic slices were collected, one to be stained only for c-Fos, one for c-Fos and orexin A, and one for c-Fos and MCH. An immunoperoxidase procedure was used to detect c-Fos as described previously [4–6].

Double labeling of c-Fos and orexin-A immunoreactivity (ir) was performed as reported previously [19]. For the detection of c-Fos and MCH co-localization, the same protocol was used except that a specific antibody against MCH was used (rabbit anti-MCH; 1:3,000, Oncogene, Cambridge, Mass., USA). To produce images of c-Fos/orexin and the c-Fos/MCH double staining, photomicrographs were taken separately under fluorescent (orexin A and MCH staining) and bright-light (c-Fos staining) illumination of the same LHA region. Then the transparency of both images was reduced to 60%, the images were overlaid and the fluorescent images were converted to grayscale images using Adobe Photoshop software.

One set of hypothalamic sections of the POMC-GFP mice was double stained for GFP and c-Fos. Immunostaining for c-Fos was conducted first, as described above. GFP was visualized by the same immunoperoxidase procedure using anti-GFP antibodies (1:5,000; Stratagene, Santa Clara, Calif., USA) and aminoethylcarbazole as chromogen (Zymed Inc., San Francisco, Calif., USA).

The localization of the immunoreactive signals was identified according to the mouse brain atlas by Hof et al. [20]. The number of c-Fos-positive cells was counted manually in a blinded fashion on 6 corresponding sections in the ARC (Br –1.6 to –2.1). Our previous results in rats [21] and our unpublished observations in mice suggested that the fasting-induced c-Fos expression occurs in the rostral LHA (orexin-A and MCH-negative area; Br –0.7 to –0.9), but not in the caudal part of the LHA (Br –1.1 to –1.3). Because it seemed that fasting might have a specific effect on distinct neuronal populations of the LHA, we quantified the c-Fos response separately in the rostral and caudal LHA on 5 sections each in both regions. In the caudal LHA the co-localization of orexin-A/c-Fos and MCH/c-Fos were also quantified.

The mean value of the cell count per section of an individual animal was used for statistical analyses. Photomicrographs were taken by a digital camera (AxioCam, Carl Zeiss AG, Göttingen, Germany).

In Situ Hybridization and Immunohistochemistry

In the transgenic POMC-GFP mice one set of hypothalamic brain sections was double-stained for NPY and c-Fos using in situ hybridization histochemistry and immunohistochemistry, respectively. The antisense (CAGAGGCACCCAGAGCAGACA-CCCGCCGCTCAGCGACGACTGCCCGCCCGCCACGATG-CTAGGTAACAAGCGAATGGGGCTGTGTGGACTGAC-CCTCGCTCTATCTCTGCTCGTGTGTTTGGGCATTCTG-GCTGAGGGGTACCCCTCCAAGCCGGACAATCC) and sense riboprobes (kind gift of Herbert Herzog, Garvan Institute of Medical Research, Sydney, Australia) were labeled with digoxigenin (DIG) according to the manufacturer's instructions (Roche, Rotkreuz, Switzerland). The in situ hybridization was conducted first, using RNAse-free solutions and glassware. The sections were air dried at room temperature, rehydrated in PBS, treated with glycine (0.1 M in PBS) for 5 min, acetylated (in 0.25% acetic anhydride for 10 min), washed in PBS and hybridized in a hybridization buffer containing 200 ng/ml DIG-labeled riboprobes (18 h at 45°C). Following post-hybridization washes and digestion with RNAse A, alkaline phosphatase-conjugated anti-DIG antibody (1:500, Roche) was applied overnight and detected in NBT/BCIP solution (Roche). After rinsing in PBS, the c-Fos immunostaining was conducted as described above, except that the DAB solution did not contain nickel and cobalt for intensifying the DAB signal. NPY mRNA and c-Fos protein co-localization was evaluated on 7 consecutive sections in the ARC.

Glucose and Hormone Determination

Before transcardial perfusion in the immunohistological experiments, blood samples were taken from the anesthetized mice by puncturing the right ventricle. Blood glucose (Glucometer Elite®, Bayer), plasma insulin and leptin (Linco Research, St. Charles, Mo., USA; mouse endocrine lincoplex kit) and ghrelin (Linco Research; ghrelin total RIA kit) levels were determined.

Statistics

Multiple group comparison was conducted using ANOVA and Student-Newman-Keuls post-hoc test. Student's t test or paired t test was used to compare two groups. $p < 0.05$ was considered significant. Results are presented as means ± SEM.

Results

Hypothalamic c-Fos Expression in Fed and 14-Hour Fasted Lean and LOO Mice

Only few c-Fos ir cells were present in the ARC of ad libitum-fed animals, with no difference between the lean and obese groups (fig. 1A–E). Fourteen hours of fasting significantly increased c-Fos expression in lean animals only ($p < 0.001$). In the ARC of fasted lean mice 93.6 ± 0.7% of the c-Fos-positive neurons contained NPY mRNA, and 79.2 ± 2.5% of all NPYergic neurons expressed c-Fos (fig. 1F). In the POMC neurons virtually no c-Fos ir was detected in the fasted lean mice (0.5 ± 0.5%; fig. 1G).

Similar to the ARC, the PVN of lean but not LOO mice contained a significantly higher number of c-Fos-positive cells after 14-hour food deprivation than when fed ad libitum (fig. 2).

In the LHA, c-Fos expression was quantified separately in its rostral and caudal regions. After fasting, c-Fos expression only increased in the rostral LHA and only in the lean mice (fig. 3A–C) compared to ad libitum-fed controls ($p < 0.001$). Under the ad libitum-feeding condition, no difference was found in the number of c-Fos-positive cells between lean and obese mice in any region (fig. 3C, D).

Orexin and MCH immunopositive neurons were only detected in the caudal LHA (fig. 4) in accordance with previous findings [22, 23]. The number of orexin-positive and c-Fos/orexin double-positive neurons was similar in all 4 groups (fig. 4C, D). In the caudal LHA very few c-Fos-positive neurons contained MCH, with no difference between the lean and obese mice under any feeding condition (fig. 4). The number of MCH-containing neurons was similar in all 4 groups (fig. 4G).

Lean mice of different ages had similarly high levels of c-Fos expression in the ARC, PVN and rostral LHA after 14 h of fasting, suggesting that age had no influence on the fasting-induced c-Fos expression. There was no statistically significant difference between any age group (3, 9, 12, 18 months; fig. 5, also compare with fig. 1–3).

Glucose and Hormone Determination and Weight of Fat Pads

Blood glucose and plasma ghrelin levels did not differ between ad libitum-fed lean and LOO mice, respectively. Insulin and leptin levels were significantly higher in the ad libitum-fed LOO animals compared to lean controls (fig. 6). After 14 h of food deprivation glucose decreased significantly only in the lean mice but not in LOO animals. Leptin and insulin levels decreased in both groups of fasted mice, but fasted LOO mice still had significantly higher values than fasted lean mice. In response to fasting, ghrelin levels increased significantly and similarly in lean and LOO mice.

Compared to the lean mice, the LOO mice had 11-fold larger perirenal (0.10 ± 0.01 vs. 1.11 ± 0.08 g) and 6-fold larger epididymal (0.35 ± 0.03 vs. 2.13 ± 0.08 g) fat pads. The fat pad weights of lean mice across the different age groups are summarized in table 1. The 12-month-old animals had the largest fat pads, associated with the highest fasting blood glucose and leptin levels. No significant differences were found in fasting ghrelin and insulin levels between the 4 age groups (table 1).

Leptin and Insulin Sensitivity

Compared to the respective saline group, leptin significantly reduced food intake in lean mice starting from 8 h following injection, while leptin was without effect in the LOO mice (fig. 7A).

Insulin significantly reduced blood glucose levels in the lean mice 30 min after injection compared to saline, while insulin had no effect on blood glucose levels in the LOO mice (fig. 7B). There was a significant difference in the blood glucose levels of insulin injected lean and obese mice 10, 20 and 30 min after injection.

Food Intake after 12-Hour Food Deprivation

Compared to ad libitum-fed controls, cumulative food intake was significantly higher after 1 and 12 h of refeeding in the young lean mice following 12 h of food deprivation. At the same time points, food intake of the LOO mice was similar regardless of whether the mice had been previously fasted or fed ad libitum (fig. 7C, D).

Respiratory Quotient and Energy Expenditure

The mean respiratory quotient was significantly lower in both fasted groups compared to ad libitum-fed groups

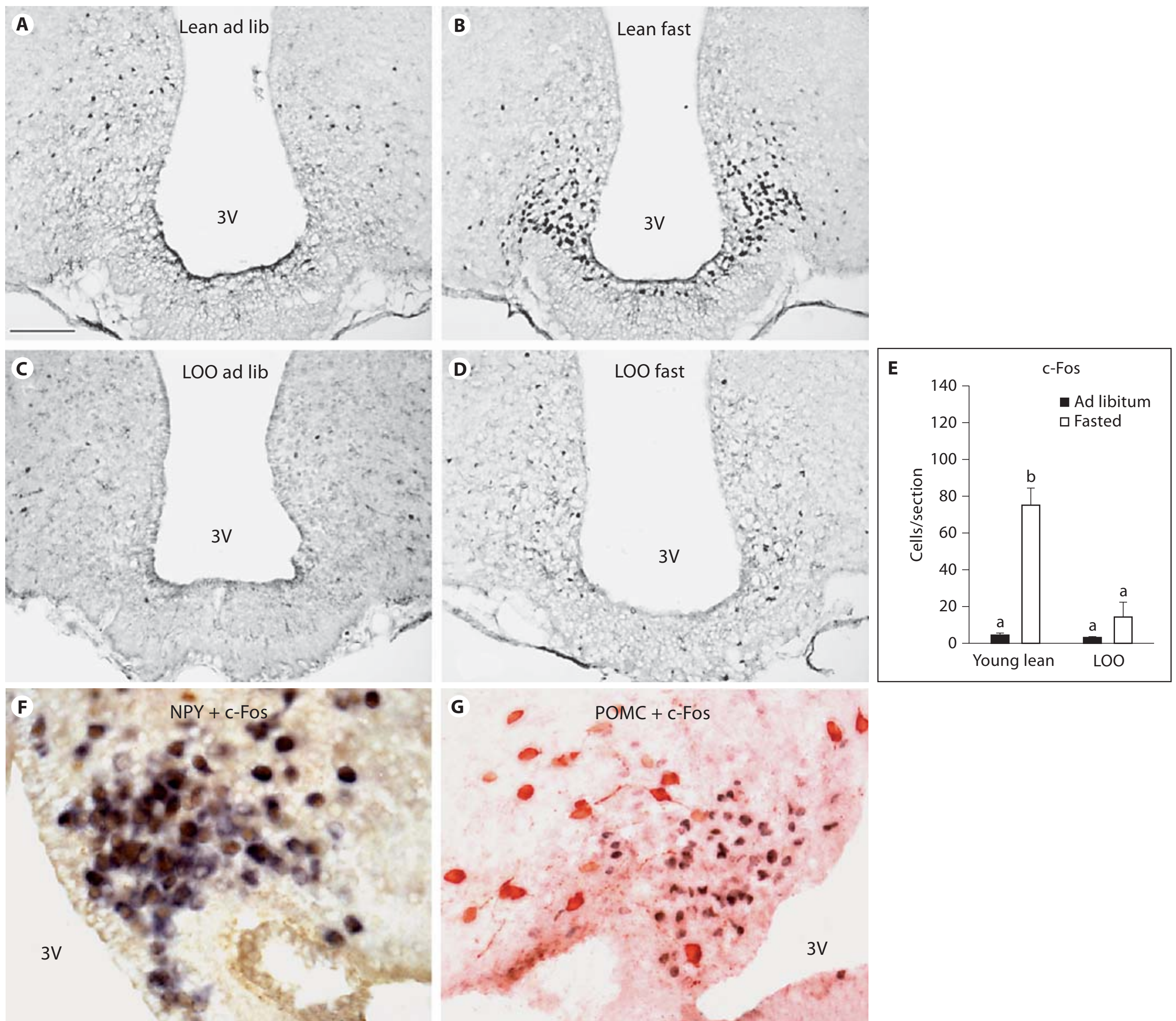

Fig. 1. Representative ARC sections immunostained for c-Fos of fed and 14-hour fasted young lean (**A**, **B**; n = 9 and 11, respectively) and LOO (**C**, **D**; n = 6/group) mice. Bar chart shows the quantitative results (**E**). Different letters indicate significant differences between groups. Representative ARC sections immunostained for c-Fos/NPY mRNA (c-Fos: brown nuclear staining; NPY mRNA: black cytoplasmatic staining; **F**) and c-Fos/POMC (c-Fos: black nuclear staining; POMC: red cytoplasmatic staining; **G**) of 14-hour fasted POMC-GFP mice. 3V = 3rd ventricle. Scale bar = 100 μm (**A–D**).

starting from 3 h after the beginning the food deprivation. There was no difference between obese and lean mice under any feeding condition (fig. 7E).

Energy expenditure was analyzed during the light phase, because in this time period locomotor activity did not differ significantly between lean and obese mice whether fed ad libitum (9.8 ± 0.8 vs. 10.9 ± 1.6 m) or fasted (13.7 ± 2.3 vs. 17.5 ± 4.6 m). Compared to ad libitum-fed controls, there was no significant difference in the percent decrease in total 12-hour energy expenditure between lean (5.84 ± 0.7%) and obese (3.58 ± 3.4%) mice during food deprivation.

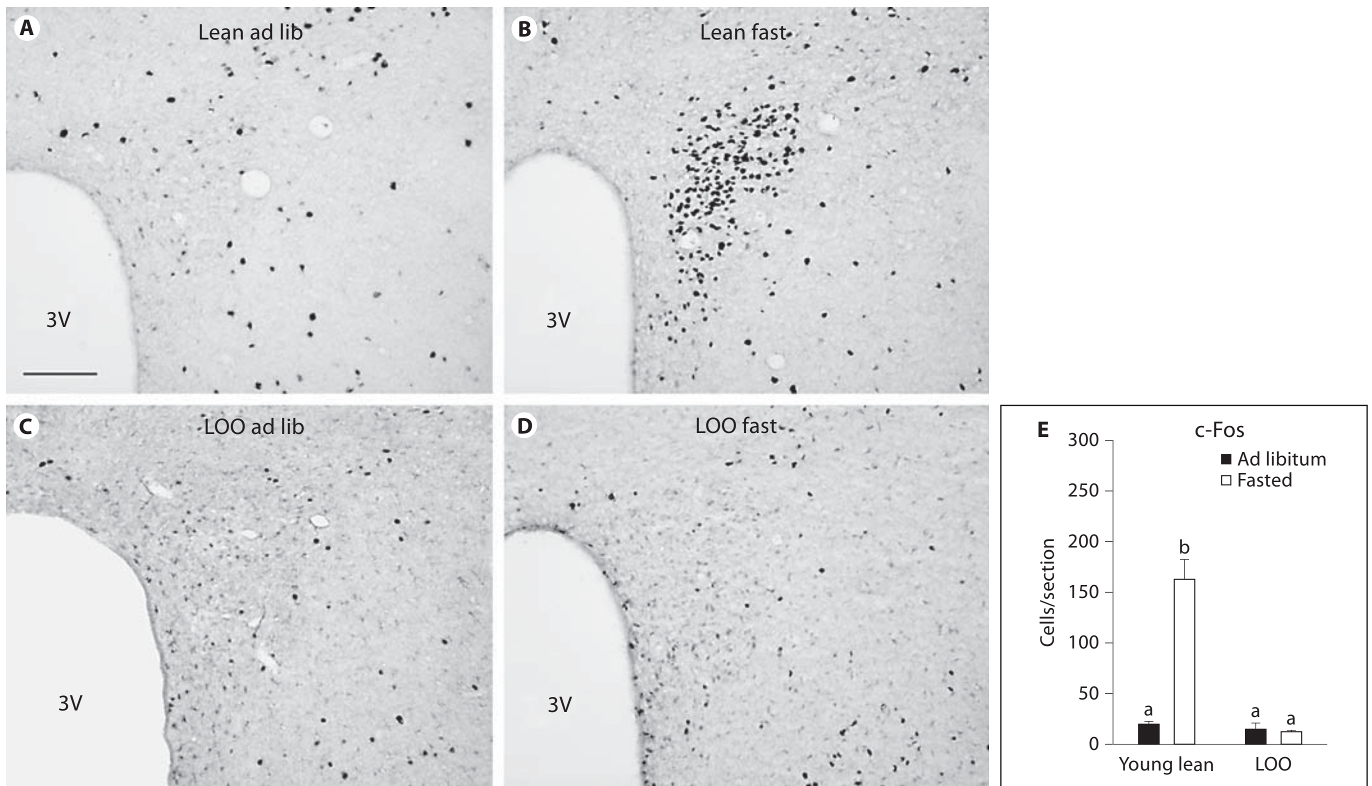

Fig. 2. Representative PVN sections immunostained for c-Fos of fed and 14-hour fasted young lean (**A**, **B**; n = 9 and 11, respectively) and LOO (**C**, **D**; n = 6/group) mice. Bar chart shows the quantitative results (**E**). Letters above the bar charts indicate the results of the statistical tests. Different letters indicate significant differences between groups. Groups with no significant differences in the given parameter bear the same letter. 3V = 3rd ventricle: Scale bar = 100 μm.

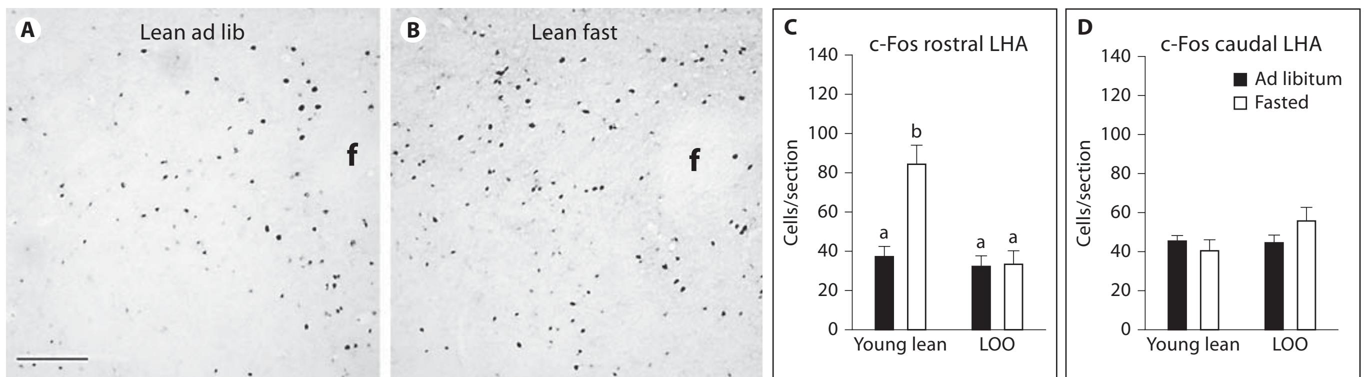

Fig. 3. Representative rostral LHA sections immunostained for c-Fos of ad libitum fed (**A**; n = 9) and fasted (**B**; n = 11) young lean mice. Number of c-Fos-positive neurons in the rostral (**C**) and caudal (**D**) LHA of lean and LOO mice. Letters above the bar charts indicate the results of the statistical tests. Different letters indicate significant differences between groups. Groups with no significant differences in the given parameter bear the same letter. f = Fornix. Scale bar = 100 μm.

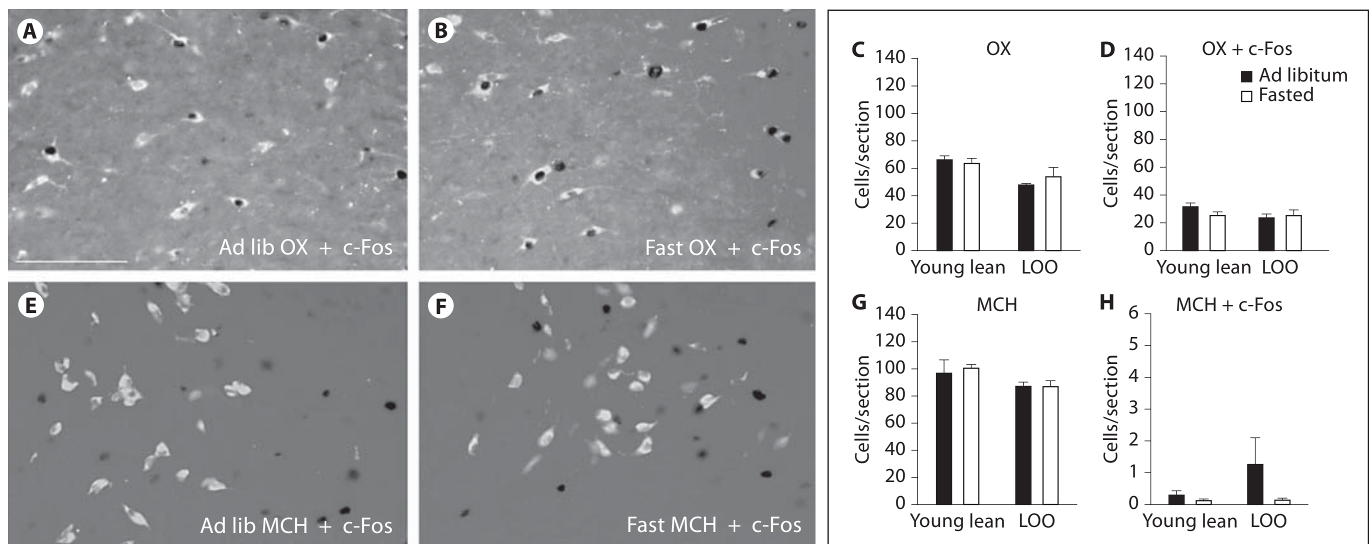

Fig. 4. Representative caudal LHA sections immunostained for c-Fos (black nuclear staining) and orexin A (OX; grey cytoplasmatic staining) of ad libitum fed (**A**) or fasted (**B**) young lean mice. Number of OX (**C**) and OX/c-Fos double-positive (**D**) neurons in the caudal LHA of lean and LOO mice. Representative caudal LHA sections immunostained for c-Fos (black nuclear staining) and MCH (grey cytoplasmatic staining) of ad libitum fed (**E**) or fasted (**F**) young lean mice. Number of MCH positive (**G**) and MCH/c-Fos double-positive (**H**) neurons in the caudal LHA of lean and LOO mice. Scale bar = 100 μm.

Table 1. Fat pad weight and blood parameters of 14-hour food-deprived lean mice of different age groups

	3 months	9 months	12 months	18 months
Body weight, g	25.7 ± 0.3^{a}	31.0 ± 0.6^{b}	33.0 ± 0.4^{c}	33.5 ± 0.6^{c}
Perirenal fat pad, g	0.06 ± 0.01^{a}	$0.15 \pm 0.02^{a, b}$	0.26 ± 0.06^{b}	$0.09 \pm 0.01^{a, b}$
Epididymal fat pad, g	0.27 ± 0.03^{a}	0.55 ± 0.07^{b}	0.73 ± 0.16^{b}	0.54 ± 0.09^{b}
Glucose, mmol/l	$7.45 \pm 0.8^{a, b}$	$8.48 \pm 1.0^{a, b}$	10.17 ± 1.2^{a}	6.45 ± 0.3^{b}
Ghrelin, ng/ml	5.72 ± 0.59	5.65 ± 0.24	5.09 ± 0.67	4.68 ± 0.79
Leptin, ng/ml	0.47 ± 0.2^{a}	1.11 ± 0.3^{a}	3.72 ± 1.4^{b}	0.75 ± 0.2^{a}
Insulin, ng/ml	0.80 ± 0.2	0.86 ± 0.5	0.95 ± 0.2	1.45 ± 0.5

Letters indicate the results of the statistical tests. Different letters indicate significant differences between groups. Groups with no significant differences in the given parameter bear the same letter.

Discussion

The ARC, LHA and PVN are key components of the central network controlling food intake and energy balance. Our present findings demonstrate that the responsiveness of this hypothalamic circuit to challenges of energy homeostasis is reduced in obese mice compared to lean controls. While 14 h of food deprivation increased neuronal activity in all three hypothalamic nuclei in lean mice, it had no such effect in obese mice. Furthermore, the lack of fasting-induced c-Fos responses in the hypothalamic nuclei of obese mice was paralleled by a lack of fasting-induced hyperphagia.

The mouse model of late-onset obesity used here resembles the most common form of human obesity in that it develops over a long time period. Furthermore, our results showed that similar metabolic and hormonal alterations were present as in obese humans and other murine obesity models, e.g. diet-induced obesity. LOO mice were hyperleptinemic, hyperinsulinemic, and had elevated blood glucose in the fasted state. Additionally, they showed impaired responses to leptin and insulin com-

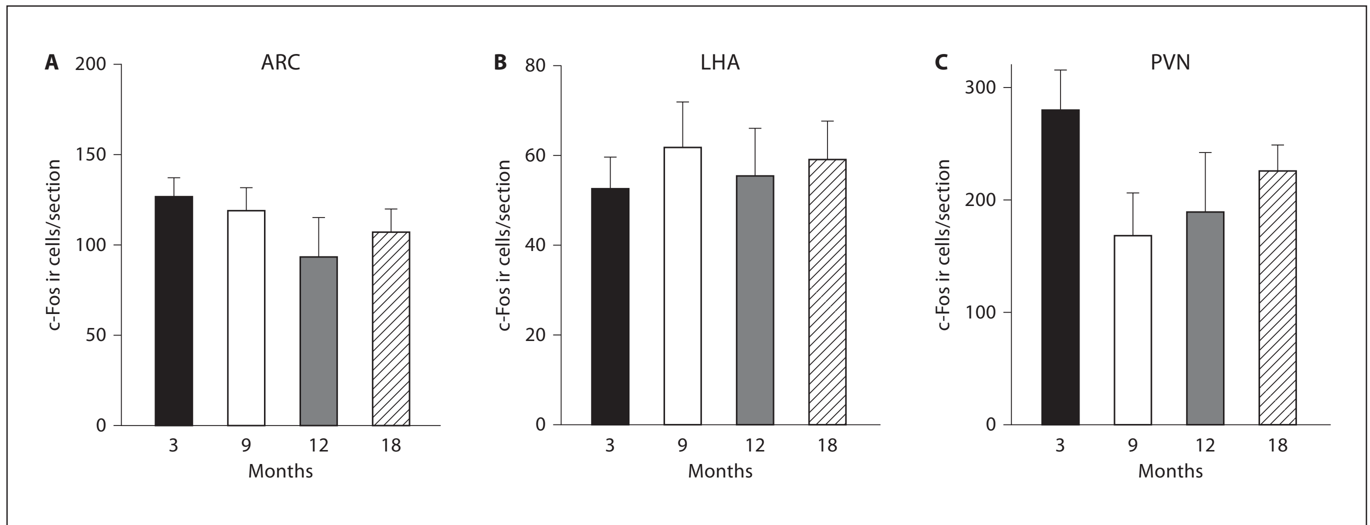

Fig. 5. Number of c-Fos-positive cells in the ARC (**A**), rostral LHA (**B**) and PVN (**C**) of 14-hour food-deprived mice of different age groups (n = 5–6).

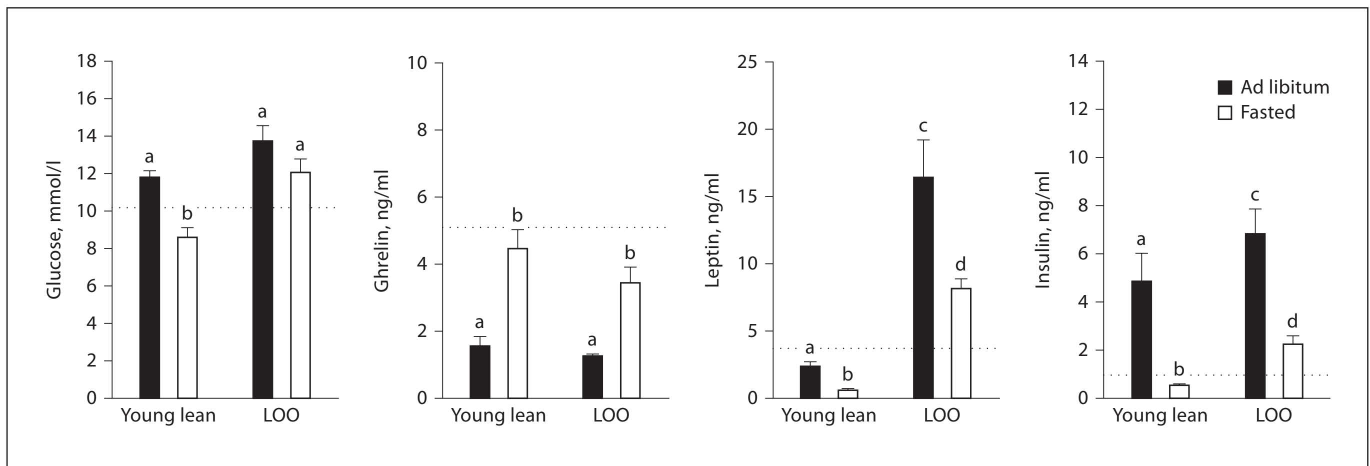

Fig. 6. Blood glucose and plasma ghrelin, leptin and insulin levels of ad libitum fed and 14-hour fasted young lean and LOO mice. Letters above the bar charts indicate the results of the statistical tests. Different letters indicate significant differences between groups. Groups with no significant differences in the given parameter bear the same letter. For comparison the mean values of these parameters of the fasted 12-month-old lean mice are shown as a dotted horizontal line.

pared to lean animals. As our experiments with lean mice of different ages showed, age does not seem to directly influence the fasting-induced neuronal activation in the LOO model. We therefore assume that the differences in the hypothalamic fasting-induced activation between lean and obese mice might be related to the altered metabolic and hormonal responses of LOO mice to short-term fasting. Interestingly, the 18-month-old lean mice appeared to have smaller fat pads and lower levels of leptin and glucose than their 12-month-old counterparts. These changes might be related to a reduction in food intake, which is a common phenomenon in humans and animals at old age [24].

In contrast to the lack of c-Fos response to fasting, our data demonstrate adaptive responses of LOO mice to negative energy balance. LOO mice showed fasting-

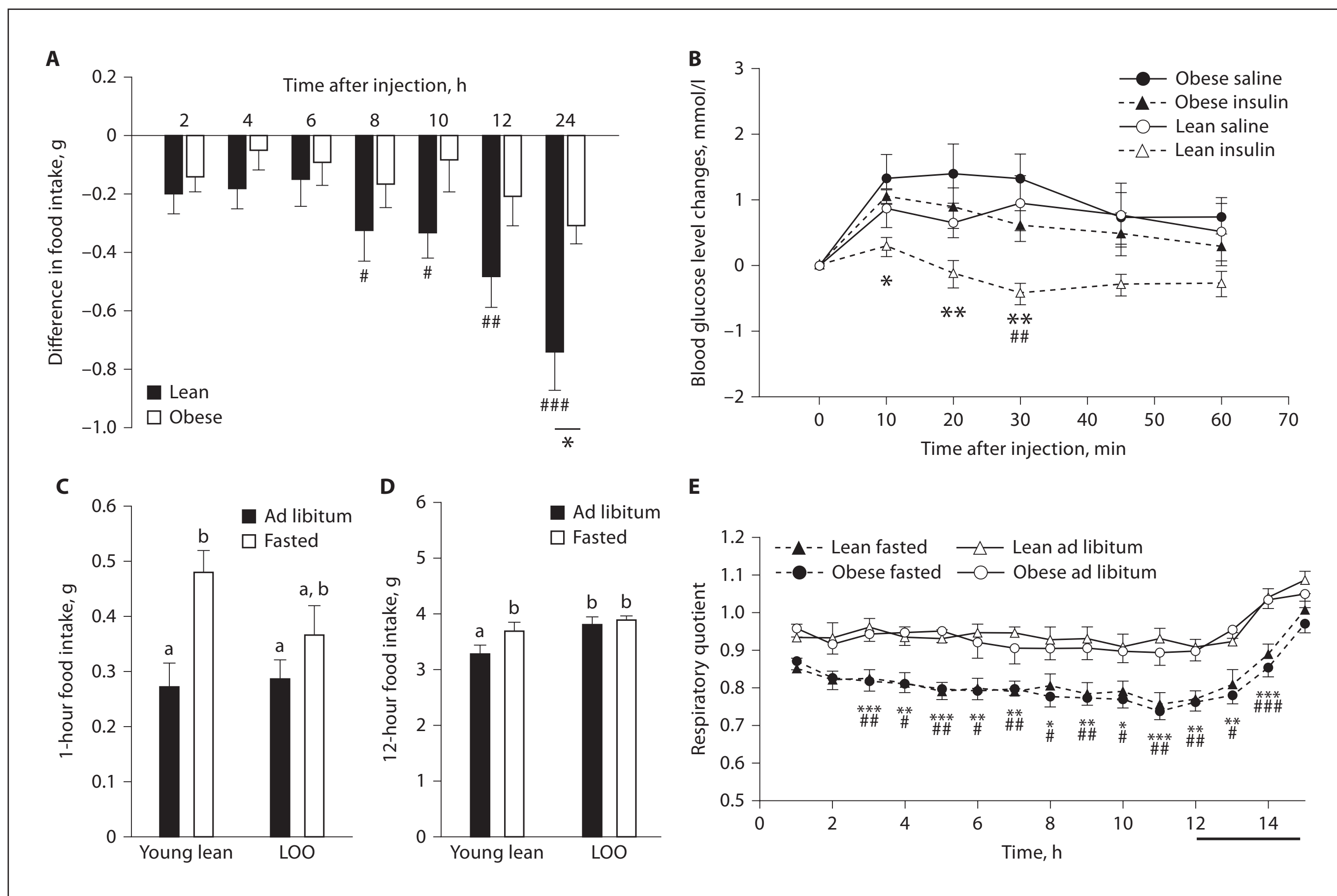

Fig. 7. A Food intake of ad libitum fed LOO and age-matched lean mice after leptin injection (2.6 mg/kg s.c.; n = 8/group). Bar charts show differences in food intake compared to saline injection. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ lean leptin vs. saline; * $p < 0.05$ lean leptin vs. LOO leptin. **B** Changes in blood glucose levels of ad libitum fed young lean (n = 6) and LOO (n = 9) mice after saline and insulin (0.01 U/kg s.c.) injection compared to pre-injection levels. * $p < 0.05$, ** $p < 0.01$ lean insulin vs. LOO insulin; ## $p < 0.01$ lean insulin vs. saline. **C**, **D** 1- and 12-hour food intake of fed and 12-hour food-deprived young lean and LOO mice (n = 8/group). Letters above the bar charts indicate the results of the statistical tests. Different letters indicate significant differences between groups. Groups with no significant differences in the given parameter bear the same letter. **E** Respiratory quotient of fed and 14-hour food-deprived LOO and age-matched lean mice (n = 6/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ lean fasted vs. ad libitum; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ LOO fasted vs. ad libitum. Black horizontal bar indicates the dark phase of the illumination cycle (starting at t = 12 h).

induced reductions in energy expenditure and shifts in substrate utilization towards increased fat utilization as reflected by the lower respiratory quotient. There were no obvious differences compared to lean mice, although a direct comparison of energy balance would require a correction for body adiposity and body temperature. Notably, our preliminary studies indicate that even more severe acute energy depletion (26 h of fasting) does not trigger a neuronal response in the ARC of LOO mice [25].

In the ARC of lean mice, the fasting-induced neuronal activation involved almost exclusively the NPYergic neurons. These neurons are well-characterized targets of circulating ghrelin, leptin, insulin and glucose [1, 7–9]. Thus, the fasting-induced changes of these factors might contribute to the activation of these NPY neurons in lean mice. Several lines of evidence underline the importance of glucose in modulating ARC activity. The NPYergic neurons are excited by decreasing glucose concentration [7]. Furthermore, in our previous study we demonstrated

that peripheral glucose application reversed the fasting-induced ARC activation in lean mice [5]. Hence, it is conceivable that in the lean mice, the fasting-induced decline in glucose levels is an important signal that contributes to the activation of the ARC, and that the elevated fasting glucose in LOO mice is likely to prevent this response. It is intriguing to speculate about the role of fasting-induced decrease in blood glucose levels in fasting-induced hyperphagia, because glucoprivation and insulin-induced severe hypoglycemia increase food intake [26, 27]. However the feeding responses to glucoprivation and food deprivation seem to be separable. As shown by Luquet et al. [28] the NPYergic ARC neurons do not seem to be involved in the first response, but they are essential for the fasting-induced hyperphagia. Furthermore, the relatively slight decrease in blood glucose levels during fasting does not appear to be a major factor in the hyperphagic response, because mice do not increase food intake when a similar reduction in glucose levels is induced by insulin (unpublished observation).

In the present study the lack of ARC activation in obese mice occurred despite a fasting-induced increase in ghrelin levels, which however was similar to that observed in lean mice. This suggests that the increase in ghrelin levels is unlikely to account for the fasting-induced ARC activation in general. This is consistent with our recent findings that neutralization of circulating ghrelin by a novel RNA-based antagonist (anti-ghrelin Spiegelmer NOX-11B-3) had no effect on the fasting-induced c-Fos expression in the ARC of lean mice under comparable experimental conditions [6].

Leptin and insulin inhibit NPYergic ARC neurons [1]. Thus in lean mice the fasting-induced decrease in leptin and insulin might reduce the inhibitory tone on the NPYergic neurons. Hence, this disinhibition may explain the activation of these neurons under fasting conditions, or it may facilitate the fasting-induced activation of these neurons by other metabolic or hormonal signals. This disinhibitory role of leptin is further substantiated by our recent studies showing an exaggerated fasting-induced c-Fos expression in the ARC of obese leptin-deficient ob/ob mice compared to lean littermates [25]. In our LOO mice, the lack of fasting-induced ARC activation was accompanied by significantly elevated fasting leptin and insulin values, compared to fasted lean mice. Therefore, it is plausible that insulin and particularly leptin levels did not decrease sufficiently in obese mice to allow disinhibition of NPY neurons in the ARC.

It might appear paradoxical to argue with a high inhibitory effect of leptin and insulin in obese animals. Obese humans and animals are generally considered resistant to certain effects of these peptides [10–12]. Similar to other obesity models, the anorectic effect of leptin and the hypoglycemic effect of insulin were also reduced in our LOO mice. Therefore, further studies are required to elucidate the impact of hyperleptinemia and hyperinsulinemia on hypothalamic activity in negative energy balance. Nevertheless, it has to be noted that leptin responsiveness may increase as a result of energy restriction. In lean animals food deprivation enhances neuronal and behavioral responses to exogenous leptin [29–31]. It remains to be investigated whether a short-term increase in leptin responsiveness may also occur under our experimental conditions in fasted LOO mice.

Similar to the ARC, food deprivation did not induce an increase in c-Fos expression in the LHA of obese mice. In the fasted lean mice, only a distinct population of neurons in the rostral LHA was activated. Although the phenotype of these cells is not yet known, it is likely that they are involved in the maintenance of energy homeostasis. Previous work from our group, showing that the fasting-induced c-Fos expression in a homologous area in rats is reversed by refeeding and by the anorectic hormone amylin, supports this notion [21]. Furthermore, similar to the ARC, the LHA also contains glucosensitive neurons which are excited when ambient glucose levels fall, e.g. in response to food deprivation [32].

Interestingly, the neuronal activity level did not change in the orexin and MCH neurons in the caudal LHA after food deprivation. In addition to the stimulatory effects on food intake, orexins and MCH also play a role in the regulation of the sleep/wake cycle. In rodents c-Fos expression in the orexinergic neurons is high in the active dark phase and low in the resting light phase, while MCH neurons show opposite responses [33–35]. Therefore, it is possible that the effects of food deprivation were masked by the influence of arousal under our experimental conditions. Arousal may have already activated a subset of the orexinergic neurons during the early dark phase, i.e. at the time when the mice were euthanized. Further, the arousal-dependent inhibition of MCH neurons at this time point may have prevented or reversed an activation of these neurons. Such mechanisms are in line with our finding that a considerable fraction of orexin neurons (approximately 50%) was activated in the ad libitum-fed mice while very few activated MCH neurons were detected in the same animals.

The ARC, PVN and LHA are interconnected by reciprocal neuronal projections [1]. Therefore, it is likely that the c-Fos responses in these nuclei to fasting partly rely

on intrinsic hypothalamic projections. This particularly applies to the PVN, which integrates inputs from numerous hypothalamic areas, and which is one of the most important hypothalamic downstream structures of the ARC. The ARC, PVN and LHA also receive inputs from extrahypothalamic areas that are involved in the control of energy homeostasis. Hence, ascending inputs from hindbrain areas might also modulate hypothalamic neuronal activity.

We conclude that, compared to lean animals, mice with late-onset obesity show blunted hypothalamic and feeding responses to the metabolic challenge of fasting. Our results demonstrate that age of LOO mice is not a critical factor causing the reduced responses in this obesity model. Our observations suggest that the pathophysiological metabolic and hormonal perturbations associated with obesity, including impaired fasting glucose, hyperleptinemia and hyperinsulinemia override the physiological neuronal responses to fasting. Therefore, a reduction of these signals seems to be necessary for the fasting-induced hypothalamic activation. Further studies are required to elucidate the underlying mechanisms and to extend our findings in other rodent models of obesity and under other experimental paradigms.

Acknowledgements

We are grateful to B.B. Lowell for providing the POMC transgenic mice, and Nina Balthasar and Elisabeth Edelstein for their help with the GFP immunostaining protocol (Beth Israel Deaconess Medical Center, Boston, Mass., USA). We thank Herbert Herzog for providing the NPY riboprobes and Nikki Lee for the superb support with the in situ hybridization (Garvan Institute of Medical Research, Sydney, Australia). The technical help of Beat Grenacher and Karin Kaufmann is deeply acknowledged (University of Zurich). C.B. was a recipient of a fellowship grant from the Zurich Centre of Integrative Human Physiology (University of Zurich) and a grant from the Research Committee and Young Academics Support Committee of the University of Zurich.

References

1 Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG: Central nervous system control of food intake. Nature 2000;404:661–671.
2 Morikawa Y, Ueyama E, Senba E: Fasting-induced activation of mitogen-activated protein kinases (ERK/p38) in the mouse hypothalamus. J Neuroendocrinol 2004;16:105–112.
3 Ueyama E, Morikawa Y, Yasuda T, Senba E: Attenuation of fasting-induced phosphorylation of mitogen-activated protein kinases (ERK/p38) in the mouse hypothalamus in response to refeeding. Neurosci Lett 2004;371:40–44.
4 Riediger T, Bothe C, Becskei C, Lutz TA: Peptide YY directly inhibits ghrelin-activated neurons of the arcuate nucleus and reverses fasting-induced c-Fos expression. Neuroendocrinology 2004;79:317–326.
5 Becskei C, Lutz TA, Riediger T: Intraperitoneal glucose application reverses the fasting-induced c-Fos expression in the hypothalamic arcuate nucleus of mice. Neuroreport 2008;19:105–109.
6 Becskei C, Bilik KU, Klussmann S, Jarosch F, Lutz TA, Riediger T: The anti-ghrelin Spiegelmer NOX-B11-3 blocks ghrelin- but not fasting-induced neuronal activation in the hypothalamic arcuate nucleus. J Neuroendocrinol 2008;20:85–92.
7 Fioramonti X, Contie S, Song Z, Routh VH, Lorsignol A, Penicaud L: Characterization of glucosensing neuron subpopulations in the arcuate nucleus: integration in NPY and POMC networks? Diabetes 2007;56:1219–1227.
8 Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S: A role for ghrelin in the central regulation of feeding. Nature 2001;409:194–198.
9 Wang L, Saint-Pierre DH, Tache Y: Peripheral ghrelin selectively increases Fos expression in neuropeptide Y-synthesizing neurons in mouse hypothalamic arcuate nucleus. Neurosci Lett 2002;325:47–51.
10 Halaas JL, Boozer C, Blair-West J: Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. Proc Natl Acad Sci USA 1997;94:8878–8883.
11 Bjorntorp P: Body fat distribution, insulin resistance, and metabolic diseases. Nutrition 1997;13:795–803.
12 Heymsfield SB, Greenberg AS, Fujioka K, Dixon RM, Kushner R, Hunt T, Lubina JA, Patane J, Self B, Hunt P, McCamish M: Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. JAMA 1999;282:1568–1575.
13 Münzberg H, Flier JS, Bjørbaek C: Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 2004;145:4880–4889.
14 Enriori PJ, Evans AE, Sinnayah P, Jobst EE, Tonelli-Lemos L, Billes SK, Glavas MM, Grayson BE, Perello M, Nillni EA, Grove KL, Cowley MA: Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell Metab 2007;5:181–194.
15 Levin BE, Govek EK, Dunn-Meynell AA: Reduced glucose-induced neuronal activation in the hypothalamus of diet-induced obese rats. Brain Res 1998;808:317–319.
16 Ryabinin AE, Wang YM, Finn DA: Different levels of Fos immunoreactivity after repeated handling and injection stress in two inbred strains of mice. Pharmacol Biochem Behav 1999;63:1143–1151.
17 Scarpace PJ, Zhang Y: Elevated leptin: consequence or cause of obesity? Front Biosci 2007;12:3531–3544.
18 Wielinga PY, Alder B, Lutz TA: The acute effect of amylin and salmon calcitonin on energy expenditure. Physiol Behav 2007;91:212–217.
19 Becskei C, Riediger T, Hernadfalvy N, Arsenijevic D, Lutz TA, Langhans W: Inhibitory effects of lipopolysaccharide on hypothalamic nuclei implicated in the regulation of food intake. Brain Behav Immun 2008;22:56–64.
20 Hof PR, Young WG, Bloom FE, Belichenko PV, Celio MR: Comparative Cytoarchitectonic Atlas of the C57BL/6 and 129/Sv Mouse Brains. New York, Elsevier, 2001.

21 Riediger T, Zuend D, Becskei C, Lutz TA: The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis. Am J Physiol Regul Integr Comp Physiol 2004; 286:R114–R122.

22 de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS 2nd, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG: The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci USA 1998; 95:322–327.

23 Skofitsch G, Jacobowitz DM, Zamir N: Immunohistochemical localization of a melanin concentrating hormone-like peptide in the rat brain. Brain Res Bull 1985;15:635–649.

24 Di Francesco V, Fantin F, Omizzolo F, Residori L, Bissoli L, Bosello O, Zamboni M: The anorexia of aging. Dig Dis 2007;25:129–137.

25 Becskei C, Lutz TA, Riediger T: Hypothalamic c-Fos expression in fasted mice decreases with obesity but not with age. Proc 10th Int Congr Obesity, Sydney, Australia, 2006.

26 Beaton JR, Feleki V, Stevenson JA: Insulin hyperphagia in rats fed a low-protein diet. Can J Physiol Pharmacol 1965;43:225–233.

27 Smith GP, Epstein AN: Increased feeding in response to decreased glucose utilization in the rat and monkey. Am J Physiol 1969;217: 1083–1087.

28 Luquet S, Phillips CT, Palmiter RD: NPY/AgRP neurons are not essential for feeding responses to glucoprivation. Peptides 2007; 28:214–225.

29 Baskin DG, Breininger JF, Bonigut S, Miller MA: Leptin binding in the arcuate nucleus is increased during fasting. Brain Res 1999;15: 154–158.

30 Baskin DG, Breininger JF, Schwartz MW: SOCS-3 expression in leptin-sensitive neurons of the hypothalamus of fed and fasted rats. Regul Pept 2000;92:9–15.

31 Morrison CD, White CL, Wang Z, Lee SY, Lawrence DS, Cefalu WT, Zhang ZY, Gettys TW: Increased hypothalamic protein tyrosine phosphatase 1B contributes to leptin resistance with age. Endocrinology 2007;148: 433–440.

32 Silver IA, Erecinska M: Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. J Neurosci 1994;14: 5068–5076.

33 Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, Yanagisawa M, Saper CB, Scammell TE: Fos expression in orexin neurons varies with behavioral state. J Neurosci 2001;21:1656–1662.

34 Akiyama M, Yuasa T, Hayasaka N, Horikawa K, Sakurai T, Shibata S: Reduced food anticipatory activity in genetically orexin (hypocretin) neuron-ablated mice. Eur J Neurosci 2004;20:3054–3062.

35 Modirrousta M, Mainville L, Jones BE: Orexin and MCH neurons express c-Fos differently after sleep deprivation vs. recovery and bear different adrenergic receptors. Eur J Neurosci 2005;21:2807–2816.