Chapter 6

Food cues do not modulate the neuroendocrine response to a prolonged fast in healthy men

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Abstract

Introduction

Dietary restriction benefits health and increases lifespan in several species. Food odorants restrain the beneficial effects of dietary restriction in *Drosophila melanogaster*. We hypothesized that the presence of visual and odorous food stimuli during a prolonged fast modifies the neuroendocrine and metabolic response to fasting in humans.

Subjects & Methods

In this randomized, cross-over intervention study, healthy young men (n=12) fasted twice for 60 hours; once in the presence and once in the absence of food-related visual and odorous stimuli. At baseline and on the last morning of each intervention an oral glucose tolerance test (OGTT) was performed. During the OGTT blood was sampled and a functional MRI scan was made.

Results

The main effects of prolonged fasting were: 1) decreased plasma thyroid stimulating hormone (TSH) and triiodothyronine (T3) levels 2) down-regulation of the pituitary-gonadal axis; 3) reduced plasma glucose and insulin concentrations, but increased glucose and insulin responses to glucose ingestion; 4) altered hypothalamic blood oxygenation level dependent (BOLD) signal in response to the glucose load (particularly during the first 20 minutes after ingestion); 5) increased resting energy expenditure. Exposure to food cues did not affect these parameters.

Conclusion

This study shows that 60 hours of fasting in young men 1) decreases the hypothalamic BOLD signal in response to glucose ingestion; 2) induces glucose intolerance; 3) increases resting energy expenditure and, 4) down-regulates the pituitary-thyroid- and pituitary-gonadal axes. Exposure to visual and odorous food cues did not alter these metabolic and neuroendocrine adaptations to nutrient deprivation.

Introduction

Dietary restriction extends life span and prevents prevalent age-related diseases like cancer and diabetes in a variety of species, including non-human primates [1]. The benefits appear to come about at least in part through metabolic and neuroendocrine adaptations to nutrient deprivation [2]. Circumstantial evidence suggests that dietary restriction may have similar effects on health and longevity in humans, probably at least in part via established endocrine and metabolic adaptations to nutrient deprivation [1].

It has recently been shown that food odorants (from live yeast) restrain the beneficial effects of dietary restriction on longevity in *Drosophila melanogaster* [3]. Moreover, mutation of a gene leading to severe olfactory defects (Orb83b) alters metabolism, enhances stress resistance and extends life span in fully fed *Drosophila* [3], corroborating other evidence that aging and longevity are regulated by olfactory neurons in *Caenorhabditis Elegans* [4].

The vomeronasal organ (VNO) is an evolutionary conserved part of the (mammalian) olfactory system, which primarily responds to nonvolatile cues to relay environmental information to the hypothalamus (in mammals), allowing subsequent adaptation of reproductive and ingestive behavior and neuro-endocrine secretion [5].The VNO primarily transmits pheromonal signals, mediating social and sexual behaviors and neuro-endocrine changes pertaining to reproduction. Limited evidence indicates that (non-pheromonal) odorous stimuli perceived by the main olfactory epithelium (MOE) can also engage hypothalamic neurons in humans [6]. The hypothalamus controls systemic glucose and lipid flux in response to circulating metabolic and hormonal cues reflecting bodily energy reserves [7]. Visual food cues also impact on hypothalamic neuronal activity in healthy humans [8], suggesting that this type of stimulus may add to the putative effects of odors on neuroendocrine and metabolic features in the current experimental context.

Here we hypothesized that visual and odorous food stimuli impact on neuroendocrine and metabolic responses to a prolonged fast in humans. Specifically, we predict that exposure to attractive visual and odorous food cues during fasting blunts the hypothalamic and metabolic adaptations that normally occur in response to nutrient dep-

rivation. Although we realize that fasting is not an ideal model of chronic (mild) restriction of calories, we use (prolonged) fasting as a *proxy*, since randomized offering of food cues to people who restrict their calories for a long time is virtually impossible. Moreover, we reasoned that if odorous cues counteract the benefits of mild restriction, they may impact the corollaries of more severe restriction to an even greater extent. We quantify neuronal activity in the hypothalamus by functional magnetic resonance imaging (fMRI); this technique has previously been used to determine hypothalamic activity in response to glucose ingestion in humans [9-11]. We chose to use total fasting instead of (prolonged) calorie restriction as a model of nutrient deprivation to maximize the endocrine effects of short term deprivation, since longer term experiments of this kind are not feasible in humans, particularly with respect to modulation of exposure to food cues. Visual food stimuli were offered in addition to odorous cues to maximize the potential impact of exposure.

Subjects and Methods

Subjects

This study was executed in accordance with the principles of the revised Declaration of Helsinki and commenced after approval of the Medical Ethics Committee of the Leiden University Medical Centre. This trial was registered in at Clinicaltrials.gov (NCT01243879). All volunteers gave written informed consent before participation. We studied 12 healthy Caucasian males who were recruited via local advertisements. All participants were between 18 and 30 years (mean 22 years), had a body mass index (BMI) ranging from 20-25 kg/m^2 (mean 22.5 kg/m^2) and had fasting serum glucose levels below 6.1 mmol/l. Subjects who used medication, smoked, suffered from anosmia, had MRI contraindications or had recently donated blood were excluded from participation.

Study design

In this randomized, controlled, cross-over intervention study, participants were exposed to two sequential interventions that consisted of 60 hours of fasting in the presence or absence of food-related stimuli. Interventions occurred in random order with a wash-out period of at least 2 weeks. In the week before the first intervention, after an overnight fast, the baseline functional magnetic resonance imaging (fMRI) scan and oral glucose tolerance test (OGTT) were performed.

On a separate day the next week, subjects were admitted to our clinical research centre at 08:00 am after an overnight fast (12 hours) for a subsequent period of 48 hours of fasting. After arrival, anthropometric measures were taken, body composition was determined and substrate oxidation was measured by indirect calorimetry. During both interventions, volunteers were allowed to drink water *ad libitum*, but were not permitted to leave the research center. During the intervention without food-related cues, participants were not allowed to talk about food or to watch food-related matters on television.

During the other intervention, subjects were exposed to visual and odorous food stimuli food from 10.00 am to 12.30 pm (eggs with bacon, grilled sandwich and coffee), from 1.00 to 3.30 pm (apple pie and banana cake), from 5.00 to 8.30 pm (French fries with meat croquettes, pizza salami and garlic bread) and from 9.00 to 10.00 pm (coffee). All food items were freshly prepared and presented to the participants. On

the last morning, food stimuli were given from 6.00 to 7.00 am (bacon, grilled sandwich and coffee).

After each 60 hour fast (with or without exposure to visual and odorous food stimuli), an OGTT and fMRI were performed simultaneously. At 7.00 am an intravenous catheter was inserted. Subjects drank 75g of glucose dissolved in 300ml of water. Blood samples were drawn at t=-15, 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. During the OGTT, hypothalamic neuronal activity was measured by fMRI from time -8 till +28 minutes.

Anthropomorphic Measurements, body composition, blood pressure, heart rate
Height, weight, body mass index (BMI), hip and waist circumference were measured according to WHO recommendations. Blood pressure was measured automatically (Omron 705IT, Kyoto, Japan) at the left arm with the subject in supine position after 5 minutes of rest. Heart rate was measured by palpation of a radial artery during one minute (in rest).

Indirect Calorimetry
Subjects were placed under the ventilated hood after 12 and 60 hours of fasting (Oxycon Beta, Mijnhardt Jaegher, Breda, The Netherlands). After voiding, the patient had to lie still with eyes closed, while staying awake, for 30 minutes. Substrate oxidation was calculated from CO2 and O2 concentrations in the exhaled air as previously described [12]. If a calculated substrate oxidation value was below zero, we used 0 instead for our statistical analysis.

MRI
fMRI scans were made using a 3.0 Tesla scanner (Philips Achieva; Philips Healthcare, Best, The Netherlands). A 14mm thick midsaggital brain slice was imaged for a total time of 38.2 minutes by T2*-weighted echo-planar imaging (repetition time 120ms, echo time 30ms, flip angle 30^0, scan matrix 256x231, FOV 208x208x14mm, 900 dynamics). We used a multishot EPI sequence with an EPI-factor of 33, with 3 signal averages (nsa). No parallel imaging was used. To diminish rotations during the scanning procedure, each participant's head was fixated with cushions inside the coil

MRI analysis

fMRI data were analyzed using FSL software (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl) [13;14]. First, imaginary and real images were calculated from the 900 magnitude and corresponding phase images that were acquired. Next, for each participant separately all magnitude images were registered (aligned) to a dynamic image that was acquired shortly after drinking had finished and that showed no motion artifacts (MCFLIRT) [15]. The resulting transformation matrix was then used to register the real and imaginary images (FLIRT). After complex averaging of every 4 images, the real and imaginary data were converted back to magnitude and phase images, resulting in 225 magnitude images to be analyzed. A region of interest was manually drawn in the hypothalamic area to calculate (with the "fslmeants" command) the average hypothalamic signal for all 225 time points. We used the anterior commissure, the mammillary bodies and the optic chiasm as border "points" of the hypothalamic region of interest. The fourth border point was estimated on the virtual square resulting from the three defined points. After drawing the regions of interest, an average was created for each minute that scanning was performed. The signal of each time point was normalized to the averaged pre-drink signal, rendering the relative signal change. The investigator was not blind to the occasion when analyzing the fMRI data, but the only subjective (i.e. not automated) analytical procedure was the definition of the hypothalamic region. Moreover, all analyses were performed twice to minimize observer bias.

Blood chemistry

Serum glucose was measured using a Modular P800 chemistry analyzer of Roche Diagnostics (Mannheim, Germany) with a total coefficient of variation (CV) of 1.7%. Insulin was measured with an immunometric assay on an automated Immulite 2500 (Siemens, Breda, The Netherlands) with an intra-assay CV 6-7.5%. Serum cholesterol, high density lipoprotein (HDL) and triglycerides (TG) were measured with a fully automated P-800 module (Roche, Almere, The Netherlands). For both TG and total cholesterol (TC) the CV was less than 2%. For HDL the CV was less than 3%. Low density lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation. Prolactin, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured using an electrochemoluminescence immunoassay (ECLIA) on a Modular E170 analyzer of Roche Diagnostics. Total CVs were less than 3.5%. Serum growth hormone (GH) was measured with a sensitive immuno-fluorometric assay

(Wallac, Turku, Finland), specific for the 22-kDA GH protein, calibrated against World Health Organization International Reference Preparation (WHO IRP) 80/505. The detection limit is 0.03 mU/liter; the interassay CV between 2% and 9% for concentrations from 0.25–40 mU/liter. Serum insulin-like growth factor 1 (IGF-1) was measured using an immunometric technique on an Immulite 2500 system (Diagnostic Products Corporation, Los Angeles, CA). The intra-assay CVs were 5.0 and 7.5% at mean plasma levels of 8 and 75 nmol/liter, respectively.

Insulin-like growth factor binding protein 3 (IGFBP-3) was measured using an immunometric technique on an Immulite 2500 system (Diagnostic Products Corporation, Los Angeles, USA). The lower limit of detection was 0.02 mg/l and inter-assay variation was 4.4 and 4.8% at 0.91 and 8.83 mg/l.

Serum free T4 (FT4) and thyroid stimulating hormone (TSH) were measured using a chemoluminescence immunoassay with a Modular Analytics E-170 system (Roche, Almere, The Netherlands). The intra-assay CVs were respectively 1.6-2.2 % and 1.3-5.0 %. Serum triiodothyronine (T3) was measured with a fluorescence polarization Immunoassay on an AxSym system (Abbott, Abbott Park, IL, USA). The CV was 2.5-9.0 %.

Dehydroepiandrosterone (DHEA) was measured by radioimmunoassay (RIA) (DSL, a Beckman Coulter Company). The detection limit was 0.012 µg/liter (0.04 nmol/l); the intra-assay CV was between 5.2% and 10.8%, the interassay CV between 5.9% and 11.7%.

Testosterone was determined by a direct RIA of Siemens Healthcare Diagnostics, total CV was approximately 15%.

Sex hormone-binding globulin (SHBG) was measured on an automated Immulite 2500 (Siemens, Breda, The Netherlands) with a total CV of 8%.

Cortisol was measured using a chemoluminescence immunoassay on a Modular Analytics E-170 system (Roche, Almere, The Netherlands). Total CV was less than 3.5 % for levels between 0.19 and 1.08 µmol/L.

Statistics

The differences between values before and after interventions were statistically evaluated by two-sided paired Student's t-tests. The impact of food cues on the response to nutrient deprivation was evaluated by subtracting values obtained after intervention from those obtained before, yielding 'delta' values. The difference between deltas observed during fasting with vs. without stimuli was subsequently evaluated by two-

sided paired Student's t-test. BOLD signals were averaged per minute and subsequently evaluated statistically by repeated measures ANOVA and post hoc tested with pairwise comparisons. All statistics were performed with SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL, United States of America).

Results

Anthropometric Measurements, heart rate & blood pressure

Body weight, BMI, hip circumference and lean body mass, were diminished to a similar extent by the prolonged fast with and without exposure to food-related stimuli (Table 1). Heart rate diminished significantly during fasting without stimuli, whereas heart rate increased when food-related stimuli were given during the fast (delta heart rate -7.3 ± 3.9 after fasting without stimuli versus 1.4 ± 3.8 after fasting with stimuli, p=0.041). The prolonged fast did not significantly alter blood pressure (Table 1).

Table 1 Anthropomorphic and Cardiovascular Measurements‡

	Before starvation without stimuli	After starvation without stimuli	Before starvation with stimuli	After starvation with stimuli
Age	22.3 ± 0.8			
Length (cm)	183 ± 2			
Body weight (kg)	75.8 ± 2.3	73.0 ± 2.2 *	75.8 ± 2.1	72.8 ± 2.1 *
BMI (kg/m^2)§	22.5 ± 0.5	21.7 ± 0.5 *	22.5 ± 0.5	21.6 ± 0.5 *
Heart Rate (bpm)	76 ± 4	69 ± 3	66 ± 3	67 ± 4
Delta Heart Rate (bpm)		-7,3 ± 3,9		1,4 ± 3,8 **
Systolic Blood pressure (mmHg)	139 ± 4	143 ± 3	138 ± 4	141 ± 6
Diastolic Blood pressure (mmHg)	78 ± 2	76 ± 2	77 ± 2	73 ± 2

‡ data are depicted as mean ± SEM (standard error of the mean)
§ BMI: body mass index
* is significantly ($p<0.05$) different before and after the starvation intervention
** is significantly ($p<0.05$) different between the two starvation paradigms
There are no significant differences between the different (with and without stimuli) starvation paradigms.

Lipids and hormones in plasma

Plasma levels of cholesterol, triglycerides and the cholesterol/HDL ratio significantly increased, whereas HDL-cholesterol decreased significantly and equally after 60 hours of food abstinence with or without stimuli. LDL-cholesterol only increased significantly after fasting without stimuli. Plasma TSH and T3 levels decreased, whereas T4 levels remained unchanged in response to the prolonged fast (Table 4). Plasma levels of LH, FSH and testosterone decreased significantly, whereas SHBG levels increased (Table 4).

Table 2 Results of OGTT: measurements of glucose, insulin and C-peptide at baseline and after the interventions‡

	Baseline#	Starvation without stimuli after intervention	Starvation with stimuli after intervention
Glucose (mmol/l)	5.20 ± 0.09	3.69 ± 0.13*	3.67 ± 0.10*
Insulin (mU/l)	4.33 ± 0.56	2.33 ± 0.19*	2.08 ± 0.08*
AUC glucose	1068 ±44	1481± 51**	1455 ± 41**
AUC insulin	3959 ± 312	8472 ± 1376**	8042 ± 1202**
Time to peak glucose	51 ± 8	101 ± 8*	104 ± 7*
Time to peak insulin	75 ± 9	113 ± 6*	120 ± 6*

‡ data are depicted as mean ± SEM (standard error of the mean)
# as measured at screening after an overnight fast
* is significantly ($p<0.025$) different from baseline
** is significantly ($p<0.01$) different from baseline
There are no significant differences between the different (with and without stimuli) starvation paradigms.

Prolactin, cortisol and IGFBP-3 levels remained unaltered. The concentrations of 2 hormones were altered significantly only in response to fasting without stimuli: DHEA levels increased, whereas IGF-1 levels decreased in this experimental context. However, the difference in response between fasting with and without food cues did not reach statistical significance for either hormone.

Oral glucose tolerance test (OGTT)

Plasma glucose and insulin concentrations reduced considerably upon prolonged fasting (Table 2). In contrast, the fast significantly increased the areas under the curves of plasma glucose and insulin concentrations in response to glucose ingestion (Figure 1). The times to peak of plasma glucose and insulin concentrations were delayed by nutrient deprivation. Exposure to odorous food cues during the prolonged fast did not affect any of these metabolic adaptations.

fMRI

The hypothalamic blood oxygenation level dependent (BOLD) signal, expressed as percentage of the averaged pre-drink values, was reduced in response to glucose ingestion compared to baseline (at several time points to a significant extent, particularly during the first 20 minutes after ingestion), irrespective of exposure to food cues during the prolonged fast (Figure 2). The prolonged fast *with stimuli* induced significant differences compared to baseline at minutes 4 ($p<0.001$), 9 ($p=0.033$), 16

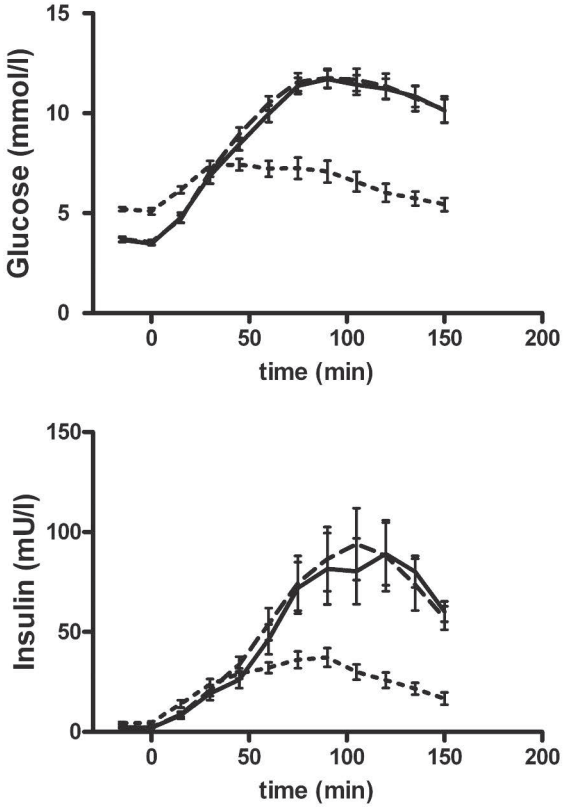

Figure 1: Plasma glucose and insulin concentrations in response to an oral glucose load at baseline and after the interventions
The dotted line represents baseline levels, the thick dotted line represents levels after the prolonged fast without stimuli and the continuous line represents levels after the prolonged fast with stimuli.

(p=0.025), 20 (p=0.025) and 27 (p=0.031). The prolonged fast *without stimuli* induced significant differences when compared to baseline at minutes 4 (p=0.012), with trends at minutes 7 (p=0.053), 20 (p=0.060), 25 (p=0.056), 26 (p=0.055). Repeated analysis confirmed these results (data not shown).

Indirect Calorimetry

Prolonged fasting significantly decreased glucose oxidation and increased lipid oxidation. There were no significant differences between the fasting paradigms (Table 3). Prolonged fasting increased resting energy expenditure significantly and consistently, irrespective of the exposure to food cues.

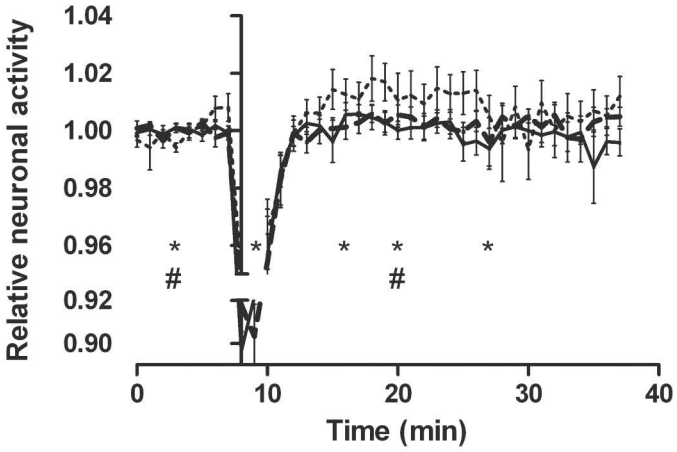

Figure 2: Hypothalamic neuronal activity in response to glucose intake at time 0 minutes as measured by functional MRI

Relative neuronal hypothalamic activity measured before and after the OGTT (started at t=8min), % change to pre-drink average is depicted.

* The prolonged fast with stimuli (continuous line) showed significant differences when compared to baseline (dotted line) at minutes 4 (p=0.000), 9 (p=0.033), 16 (p=0.025), 20 (p=0.025) and 27 (p=0.031).

The prolonged fast without stimuli (thick dotted line) showed significant differences when compared to baseline (dotted line) at minutes 4 (p=0.012), with trends at minutes 7 (p=0.053), 20 (p=0.060), 25 (p=0.056) and 26 (p=0.055)

Table 3 Substrate oxidation rates at baseline and after 3 days of starvation with or without the presence of food-related stimuli‡

	Before starvation without stimuli	After starvation without stimuli	Before starvation with stimuli	After starvation with stimuli
Glucose Oxidation (μmol/kg_{FFM}/min)	12.3 ± 1.5	7.9 ± 2.3[α]	12.6 ± 2.6	4.0 ± 1.0*
Lipid Oxidation (μmol/kg_{FFM}/min)	1.4 ± 0.1	2.3 ± 0.3*	1.5 ± 0.2	2.7 ± 0.3*
Resting Energy Expenditure (kcal/day)	1631 ± 51	1829 ± 71*	1696 ± 51	1895 ± 103*
Resting Energy Expenditure (kcal/day/LBM)	25.6 ± 0.7	30.5 ± 0.9*	27.2 ± 0.7	31.9 ± 1.3*

‡ data are depicted as mean ± SEM (standard error of the mean)
[α] p=0.076
* is significantly ($p<0.05$) different before and after the starvation intervention
There are no significant differences between the different (with and without stimuli) starvation paradigms.

Table 4 Plasma metabolites and hormones at baseline and after the interventions‡

	Before starvation without stimuli	After starvation without stimuli	Before starvation with stimuli	After starvation with stimuli
Total cholesterol (mmo/l)	4.22 ± 0.18	4.42 ± 0.20 *	4.25 ± 0.18	4.39 ± 0.19
Triglycerides (mmol/l)	0.94 ± 0.08	1.33 ± 0.11 *	0.98 ± 0.13	1.36 ± 0.11 *
HDL (mmol/l)	1.42 ± 0.08	1.25 ± 0.07 *	1.43 ± 0.06	1.25 ± 0.06 *
Cholesterol/HDL	3.06 ± 0.19	3.63 ± 0.19 *	3.05 ± 0.22	3.58 ± 0.23 *
LDL (mmol/l)	2.38 ± 0.15	2.58 ± 0.19 *	2.38 ± 0.21	2.54 ± 0.21
FT4 (nmol/l)	16.53 ± 0.60	16.60 ± 0.70	16.67 ± 0.37	17.18 ± 0.60
T3 (nmol/l)	1.82 ± 0.08	1.14 ± 0.08 *	1.91 ± 0.06	1.28 ± 0.04 *
TSH (mU/l)	2.92 ± 0.54	1.26 ± 0.19 *	2.47 ± 0.37	1.25 ± 0.18 *
Prolactin (µg/l)	13.4 ± 1.25	14.18 ± 2.79	10.93 ± 1.66	11.23 ± 1.59
DHEA (nmol/l)	10.20 ± 0.78	12.30 ± 1.26 *	11.99 ± 1.56	12.88 ± 1.31
hGH (mU/l)	4.63 ± 3.25	11.74 ± 5.19	2.11 ± 1.23	7.97 ± 2.38 [α]
IGF-1 (nmol/l)	27.30 ± 1.46	23.01 ± 1.40 *	25.05 ± 2.99	23.79 ± 1.59
IGFBP-3 (mg/l)	4.32 ± 0.18	4.33 ± 0.31	6.72 ± 2.74	4.18 ± 0.28
Cortisol (µmol/l)	0.51 ± 0.03	0.55 ± 0.04	0.53 ± 0.04	0.49 ± 0.05
FSH (U/l)	4.0 ± 0.6	3.2 ± 0.5*	4.1 ± 0.6	3.3 ± 0.5*
LH (U/l)	5.0 ± 0.3	3.3 ± 0.4*	5.1 ± 0.2	3.2 ± 0.4*
SHBG (nmol/l)	25.3 ± 1.9	28.3 ± 2.1*	24.7 ± 1.8	28.0 ± 1.7*
Testosterone (nmol/l)	28 ± 2.5	14.4 ± 1.8*	27 ± 1.6	14.7 ± 1.3*

‡ data are depicted as mean ± SEM (standard error of the mean)
* is significantly ($p<0.05$) different before and after the starvation intervention
[α] $p= 0.06$
There are no significant differences between the different (with and without stimuli) starvation paradigms.

Discussion

This study shows that 60 hours of food deprivation in healthy young men 1) alters the hypothalamic BOLD signal in response to glucose ingestion; 2) induces profound glucose intolerance; 3) down-regulates the pituitary-thyroid and pituitary-gonadal axes; and 4) increases resting metabolic rate. Exposure to (attractive) visual and odorous food cues does not alter any of these metabolic and neuroendocrine adaptations to nutrient deprivation.

This is the first study to show that prolonged fasting alters the BOLD signal produced by the hypothalamus in response to an oral glucose load in healthy humans. Since BOLD signals reflect neuronal activity [16], the data suggest that a prolonged fast renders the hypothalamic neurons relatively insensitive to glucose ingestion. It is conceivable that alterations of hypothalamic neuronal activity are an integral part of the systemic adaptations required for survival during nutrient deprivation. The hypothalamus plays a critical role in the control of (postprandial) metabolism; it integrates metabolic and hormonal cues reflecting the bodily energy status to produce neuroendocrine output, adapting energy metabolism accordingly [17]. For example, neuropeptide Y (NPY, a well-known orexigenic neuropeptide) gene expression is strongly up-regulated in the arcuate nucleus and paraventricular nucleus of fasting rhesus macaque monkeys and food-restricted type I diabetic rats [18;19]. Hypothalamic NPY induces hepatic insulin resistance via activation of sympathetic neural efferents [20]. Other hypothalamic neuronal circuits are also sensitive to nutritional cues and contribute to proper (postprandial) metabolic control [21]. Therefore, the neuronal changes in response to nutrient deprivation we observed here may be involved in the physiology of the glucose intolerance of our fasting volunteers. Insulin resistance is an appropriate metabolic adaptation to nutrient deprivation, as it renders incidentally consumed glucose available for combustion by the brain (which is largely dependent on glucose as fuel). Since nutrients tend to modulate hypothalamic neural circuits so as to reinforce insulin action [22], resistance to the neuronal effects of nutrient ingestion may be advantageous in terms of brain energetics and survival during prolonged periods of food deprivation.

The greatest effect of the oral glucose tolerance test on plasma values of glucose and insulin are seen at 50 and 75 minutes after glucose ingestion respectively,

whereas fMRI signals respond within 10-20 minutes (table 2, figure 1). These observations suggest that neither plasma glucose nor insulin is involved in the neuronal response to glucose ingestion. This inference is supported by previous work suggesting that neuronal signals emanating from the gut rather than plasma metabolite concentrations are critical for the hypothalamic response to nutrient ingestion [23].

Although we have used fMRI to quantify hypothalamic neuronal activity in similar experimental settings before [11], it seems important to note that the use of this imaging technique to measure neuronal activity in response to a single (non-iterative) stimulus is relatively uncommon and bears some difficulties. Perhaps the most important pitfall in our analysis is the rather subjective determination of the region of interest (designating the hypothalamus in the current study): small differences may significantly impact the average signal. To preclude observer bias as much as possible, the same author (M.A.W.), performed all analyses of BOLD signal changes twice, yielding similar results.

The fall of circulating triiodothyronine and TSH levels in the face of relatively stable free T4 concentrations are typical physiological adaptations of the pituitary-thyroid axis to short term fasting in humans [24;25]. These changes, which may serve to dampen basal energy expenditure, partly result from an adaptive mechanism driven by diminished activity of leptin sensitive hypothalamic TRH neurons [25]. However, despite these apparent effects of nutrient deprivation on thyroid axis activity, 60 hours of fasting paradoxically *increased* resting energy expenditure by ~11% in our volunteers. We are not the first to report this counterintuitive observation: a few previous studies similarly showed that the resting metabolic rate (RMR) of healthy humans is increased by 6 and 4% after 36 or 48 hours of food deprivation respectively [24;26]. The increase in RMR during short-term fasting might be due to the energy costs of a temporary increase in gluconeogenesis and ketogenesis [24]. Activation of the sympathetic nervous system may modify RMR during prolonged fasting [27]. In apparent contrast, RMR is clearly reduced after longer term calorie restriction and weight loss in obese humans [28], but this is probably due to the significant loss of lean body mass in this context [29].

Plasma levels of FSH, LH and testosterone significantly decreased upon fasting, while SHBG concentrations increased, which confirms the findings of other studies [30]. Long

term dietary restriction over the course of years also down-regulates the pituitary-gonadal axis in members of the Calorie Restriction Society [31]. These endocrine adaptations probably serve to postpone reproduction until food is available again. Reduction of plasma leptin levels may be mechanistically involved, since leptin administration during a prolonged fast prevents changes in testosterone levels (but not LH and SHBG) in healthy men [25].

We also show that fasting increases circulating dehydroepiandrosterone (DHEA) levels (significantly in the group that was not exposed to food-related stimuli). DHEA and its sulfate ester DHEAS are markers of human aging, since these adrenal steroids reach peak levels in the second decade of life and then gradually decline [32]. Moreover, high plasma DHEA concentrations correlate with longevity and survival in men but not in women [33]. DHEA may have beneficial effects on inflammation, cell growth, oxidative stress, carcinogenesis and atherosclerosis, possibly via inhibition of glucose-6-phosphate dehydrogenase [34]. However, longer term restriction of calories has been reported to leave plasma DHEA(S) concentrations unaffected in humans [31] and DHEAS supplements fail to extend lifespan or prevent chronic disease in rodents [35]. Therefore, the biological significance of the rise of plasma DHEA level we observed is uncertain. We did not find an effect of the prolonged-fast on cortisol levels, which may have been caused by the relatively high levels of cortisol at baseline, perhaps induced by stress due to the insertion of the intravenous catheter.
Exposure to odorous and visual food cues did not modify any of the above mentioned metabolic and neuroendocrine adaptations to a prolonged fast. It may be that olfactory cues do not impact on neuroregulatory mechanisms in humans. The olfactory system in higher mammals, including humans, comprises the olfactory bulb, the orbitofrontal cortex and the hypothalamus among other tertiary relay stations. A small number of *in vivo* fMRI studies show that odorous stimuli can alter hypothalamic neuronal activity in humans [6]. In our study, the presence of olfactory stimuli during fasting did not alter hypothalamic neuronal activity in response to a subsequent oral glucose load (during which no visual or odorous cues were offered). Although intuitively unlikely, the exposure time (9.5h/day in total) we used may have been too short or the intensity of the odors may have been too weak to bring about significant effects. Furthermore, the impact of fasting on the regulation of metabolism may be too strong to allow significant effects of modulatory processes of lesser power. Indeed, the evidence in lower organisms indicates that olfactory cues restrain the beneficial corol-

laries of calorie restriction, not total starvation. Accordingly, *Drosophila* species were calorie restricted, not starved, in the study by Libert *et al*. [3] In yeast (*Saccharomyces cerevisiae*), severe calorie restriction engages regulatory pathways to extend life span fundamentally different from those activated by more modest restriction [36;37]. In analogy, in humans (obese humans, we are unaware of studies in lean humans), modest restriction of calories does not affect insulin action in the short term [38], whereas total fasting considerably hampers insulin action in a similar timeframe [39], suggesting that the degree of dietary restriction differentially affects regulatory mechanisms in humans as well. Thus, in contrast to calorie restriction, fasting may be too strong a stimulus for metabolic adaptation to allow olfactory cues to modulate the changes.

In conclusion, prolonged fasting changes the hypothalamic neuronal response to glucose ingestion in healthy normal weight humans, which may guide postprandial endocrine and metabolic adaptations to nutrient deprivation that are meant to shunt any incidentally consumed carbohydrates towards the brain. Indeed, 60 hours of total nutrient deprivation elicits profound glucose intolerance, most likely because it hampers insulin action. Prolonged fasting also down-regulates the pituitary-gonadal and -thyroid axes, probably to appropriately adapt fecundity and energy expenditure to nutrient scarcity. Food-related olfactory cues do not modulate these neuroendocrine and metabolic adaptations to a prolonged fast in humans.

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