Impaired hypothalamic Fto expression in response to fasting and glucose in obese mice

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Objective: Recent genome-wide association studies have identified a strong association between obesity and common variants in the fat mass and obesity associated (FTO) gene. FTO has been detected in the hypothalamus, but little is known about its regulation in that particular brain structure. The present study addressed the hypothesis that hypothalamic FTO expression is regulated by nutrients, specifically by glucose, and that its regulation by nutrients is impaired in obesity.

Research design and methods: The effect of intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) administration of glucose on hypothalamic Fto mRNA levels was examined in fasted mice. Additionally, the effect of glucose on Fto mRNA levels was also investigated ex vivo using mouse hypothalamic explants. Lastly, the effect of i.p. glucose injection on hypothalamic Fto immunoreactivity and food intake was compared between lean wild-type and obese ob/ob mice.

Results: In wild-type mice, fasting reduced both Fto mRNA levels and the number of Fto-immunoreactive cells in the hypothalamus, whereas i.p. glucose treatment reversed this effect of fasting. Furthermore, i.c.v. glucose treatment also increased hypothalamic Fto mRNA levels in fasted mice. Incubation of hypothalamic explants at high glucose concentration increased Fto mRNA levels. In ob/ob mice, both fasting and i.p. glucose treatment failed to alter the number of Fto-immunoreactive cells in the hypothalamus. Glucose-induced feeding suppression was abolished in ob/ob mice.

Conclusion: Reduction in hypothalamic Fto expression after fasting likely arises at least partly from reduced circulating glucose levels and/or reduced central action of glucose. Obesity is associated with impairments in glucose-mediated regulation of hypothalamic Fto expression and anorexia. Hypothalamic Fto-expressing neurons may have a role in the regulation of metabolism by monitoring metabolic states of the body.

Keywords: hypothalamus; obesity; nutrient; gene expression; feeding

Introduction

Recent genome-wide association studies have identified a strong association between obesity and common variants in the fat mass and obesity-associated (FTO) gene.¹ Individuals homozygous for the risk allele in diverse ethnic backgrounds have increased adiposity compared with those devoid of the risk allele.² Despite the association between FTO variants and obesity, the biological function of FTO and the mechanism by which FTO variants lead to obesity are not well understood. The majority of studies currently suggest that increased energy intake, but not reduced energy expenditure, may contribute to the increased adiposity and body weight in individuals with the FTO risk alleles.² While it appears that most of FTO variants are located within the first two introns and exon 2, it remains unclear whether the obese phenotype in individuals carrying the FTO risk alleles is due to loss-of-function of FTO or if FTO itself has a role in the regulation of energy balance. Recent studies using mouse models with a complete absence of Fto or reduced Fto expression demonstrated that these mutant mice exhibit reduced body weight and adiposity compared with wild-type mice.³,⁴ Conversely, systemic overexpression of Fto increases food intake and body weight in mice, supporting the role for Fto in the regulation of metabolism.⁵

FTO is expressed in various tissues, including the hypothalamus where its levels are especially high.¹,⁶–⁸ Hypothalamic Fto mRNA levels are altered by fasting, and hypothalamic Fto-expressing cells are activated by feeding.⁹–¹⁰ Of particular interest, contrary to the metabolic phenotypes of Fto-deficient mice and Fto-overexpressing mice, targeted reduction of Fto expression in the hypothalamic arcuate...
nucleus (ARC) increased food intake and body weight, while enhanced Fto expression in ARC produced the opposite effect. These findings support the hypothesis that Fto expression is regulated by metabolic states, and that enhanced hypothalamic Fto expression promotes a negative energy balance, implicating Fto in the regulation of energy balance in a tissue-specific manner. However, little is known about the mechanisms governing the regulation of hypothalamic Fto expression and how specific nutrients may contribute to fasting-induced and feeding-induced changes in its expression.

Hypothalamic neurons contribute to the metabolic regulation by altering their own activities or the activities of their downstream targets in response to hormonal and nutrient signals. In particular, hypothalamic glucose sensing and metabolism has a critical role in the regulation of food intake, energy expenditure, and carbohydrate and lipid metabolism. The importance of hypothalamic glucose sensing in the regulation of energy homeostasis has been supported by the findings that hypothalamic responses to glucose stimulation are attenuated in obesity. Therefore, we hypothesized that hypothalamic Fto expression is regulated by glucose and this regulation is impaired in obesity.

Materials and methods

Animals
Male C57BL/6 mice were obtained from Charles River Laboratories (Montreal, Quebec, Canada). Male wild-type and ob/ob mice (C57BL/6 background) were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Animals were individually housed under a 12:12 light/dark cycle (lights on at 0600 h) with free access to standard rodent chow pellets (Prolab RMH 3000, 4.5% fat by weight,Ralston Purina) except for during fasting and feeding studies. Water was available throughout the experiment. All studies were approved by the Institutional Animal Care and Use Committee (University of Manitoba and Mount Sinai School of Medicine).

Fasting and intraperitoneal glucose treatment
Mice were fasted for 24, 30 or 48 h. Control mice were fed ad libitum throughout the experiment. Mice were killed by CO₂ narcosis followed by decapitation. Trunk blood was collected, blood glucose level was immediately measured, and serum was saved and stored at −80°C for hormone assays. The brain was quickly removed and the hypothalamus and the cerebral cortex were dissected out, immediately frozen on dry ice and stored at −80°C until RNA analysis. Mice fasted for 30 h received a single intraperitoneal (i.p.) injection of saline or glucose (2 mg/g body weight (b.w.)) at the end of the fasting period. Control mice were fed ad libitum throughout the experiment and injected i.p. with saline. Mice were killed 1 h after the i.p. injection, and the blood and tissues were collected as described above. A second set of mice received the same treatment except that the mice were perfused as described below 2 h after the i.p. injection. The brain was collected for immunohistochemical analysis. In a third set of animals, wild-type and ob/ob mice were assigned randomly to three groups as above (ad libitum fed/saline-treated, fasted/saline-treated and fasted/glucose-treated) in each genotype except that the duration of fasting was 48 h in this study. Mice were perfused as described below 2 h after the i.p. injection, and the brain was collected for immunohistochemical analysis.

Fasting and intracerebroventricular glucose injection
Mice were implanted with an intracerebroventricular (i.c.v.) cannula into the lateral ventricle as described previously. Mice received i.c.v. injection of glucose (100 μg in 1 μl) or saline every 6 h during the 30-h fast (five injections in total). The 100 μg dose was chosen because a single i.c.v. injection of glucose at this dose did not cause significant changes in serum glucose and insulin levels in mice. Saline was used as a control vehicle instead of artificial cerebrospinal fluid (aCSF) because aCSF contains glucose. Mice were killed 1 h after the i.c.v. injection and tissue collection was performed as above.

Ex vivo study
To determine the effect of glucose on hypothalamic Fto expression, hypothalamic explants from the mouse were cultured in the presence of low (1 mm) or high (10 mm) glucose. Seven ad libitum-fed male C57BL/6 mice were euthanized by exposing to isoflurane followed by decapitation in the late light cycle (between 1500 h and 1530 h). Medial basal hypothalamus was excised from the brain using the following landmarks: optic chiasm (rostral), mammillary bodies (caudal), optic tract (lateral) and apex of the hypothalamic third ventricle (superior). The excised hypothalamic tissue was split symmetrically into left and right halves at the hypothalamic third ventricle. Cerebral cortex (including cingulate/retrosplenial, motor, somatosensory, retrosplenial agranular and retrosplenial granular cortex) was also excised and split in half at the midline. Each half of the tissue from the same animal was immediately cultured in Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum, 1% penicillin/streptomycin and 1 or 10 mM glucose at 37°C with 5% CO₂. Explants were harvested after a 2-h culture and stored at −80°C until RNA analysis.

Feeding study
Wild-type (+/+) and ob/ob mice were adapted to a liquid diet (F05145P, BIO-SERV, Frenchtown, NJ, USA). Mice were fasted for 8 h during the light period in the diurnal cycle and injected i.p. with glucose (2 mg/g b.w.), cholecystokinin (CCK-8, Sigma-Aldrich, St Louis, MO, USA, 10 μg/kg b.w.)
or saline immediately before commencement of the dark cycle. *Ad libitum*-fed mice were injected i.p. with 2-deoxy-D-glucose (2-DG, Sigma-Aldrich, 500 mg/kg b.w.) or saline at 1200 h. Food intake was measured for a period of 30 min after glucose and CCK-8 treatment, and for 2 h after 2-DG treatment. The doses used in the present study have been shown to be effective in altering food intake in the mouse strain used here.24

**RNA analysis**

Total RNA was extracted from the tissue of individual animal and mRNA expression levels were measured by real-time PCR using specific primers (Supplementary Table 1), as described previously.25 Levels of mRNA were normalized to β-actin or cyclophilin mRNA levels, and are expressed as means (% of the control group) ± standard error of mean (s.e.m.). All reactions were performed in triplicates and the coefficient of variation was <5% for each triplicate.

**Immunohistochemistry**

Mice were deeply anesthetized with i.p. injection of avertin (8 mg/g b.w.) and perfused transcardially with ice-cold heparinized 0.1 mol/l phosphate buffered saline (PBS, pH 7.3) followed by ice-cold 4% (w/v) paraformaldehyde (Sigma-Aldrich) in 0.2 mol/l PBS. Mice were decapitated, the brains were removed and stored at 4 °C in 2% (w/v) paraformaldehyde (Sigma-Aldrich) in 0.2 mol/l PBS. Sections were permeabilized with 0.01 mol/l PBS containing 0.5% (v/v) Triton-X-100 for 30 min at room temperature. Sections were washed in 0.01 mol/l PBS for 3 h and stored at 4 °C for 16 h before sectioning. Transverse sections (30 µm) were cut on a cryostat and preserved as free-floating sections in cryoprotectant (0.1 mol/l PBS, 30% [w/v] sucrose, 1% [w/v] polyvinylpyrrolidone and 30% [v/v] ethylene glycol) and stored at −20 °C until use for immunohistochemistry. Cryosections were washed in 0.01 mol/l PBS for 3 h and permeabilized with 0.01 mol/l PBS containing 0.5% (v/v) Triton-X-100 for 30 min at room temperature. Sections were incubated with a polyclonal rabbit or guinea pig anti-Fto (1:2500) diluted in 0.1 mol/l PBS containing 2% (w/v) bovine serum albumin overnight at 4 °C.3 Sections were then washed three times for 1 h in 0.01 mol/l PBS followed by a 2-h incubation with a Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or a fluorescein isothiocyanate (FITC)-conjugated donkey anti-guinea pig IgG (1:200, Jackson ImmunoResearch Laboratories) at room temperature. Sections were then washed three times for 15 min in 0.01 mol/l PBS, placed on slides and mounted using an aqueous antifade mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Fluoro Gell II with DAPI, Electron Microscopy Sciences, Hatfield, PA, USA). Omission of primary antibody produced no immunoreaction.

**Histological quantification**

Immunohistochemistry was performed in at least two sections per animal covering the ventromedial nucleus (VMN), ARC, the dorsomedial nucleus (DMN) and the lateral periaqueductal area (LPA) of the hypothalamus. Immunofluorescent images were viewed using a Zeiss Axioskop2 fluorescence microscope with image capture using Axiovision 3.0 software (Carl Zeiss Canada, Toronto, Ontario, Canada). The same scanning parameters and exposure times were used for all images taken from sections involving comparisons between treatment groups. Images were adjusted for contrast to remove empty pixels by applying the same parameters for each chromophore (Photoshop Ver. 6.0, Adobe Systems, San Jose, CA, USA). Numbers of Fto-immunoreactive cells were counted in the VMN, ARC, DMN and LPA using the NIH ImageJ software (Ver. 1.43, NIH, Bethesda, MD, USA). The sum of Fto-positive cells on both sides of the brain was calculated. The counts in the two sections were averaged in each animal and used for statistical analysis.

**Blood chemistry**

Blood glucose levels were measured using a glucose meter (ELITE XL, Bayer HealthCare, Mishawaka, IN, USA). Serum concentrations of insulin and leptin were measured by enzyme-linked immunosorbent assay with commercial kits from Millipore (St Charles, MO, USA) or Mercodia AB (Uppsala, Sweden) and R&D Systems Inc. (Minneapolis, MN, USA), respectively.

**Statistical analysis**

Means ± s.e.m. of all the animals in each group were calculated. Statistical analyses were performed by one-way analysis of variance followed by Tukey–Kramer or Dunnnett’s post hoc test. Comparisons between two different treatment groups were performed by Student’s t-test. Kruskal–Wallis test was performed and the Bonferroni-corrected Wilcoxon test was used for post hoc analysis when the data were not normally distributed. Comparisons between two treatment groups were performed by Wilcoxon test when the data were not normally distributed. Correlation analyses were performed between blood glucose levels and Fto expression. Differences were taken to be significant if P < 0.05. Part of body weight and blood chemistry data (Figures 2a–d) were reported previously to address a separate hypothesis.26

**Results**

**Hypothalamic Fto mRNA levels after fasting**

Fasting for 24 h, 30 h and 48 h significantly reduced hypothalamic Fto mRNA levels by 53%, 54% and 50%, respectively, compared with the *ad libitum*-fed condition (Figures 1a and e). Blood glucose, serum insulin and leptin levels were significantly reduced after the 30-h fast (Figures 1b–d). Fto mRNA levels in the cerebral cortex, measured as a control only at the 30 h fast period, were not affected (P = 0.19 by Student’s t-test, Figure 1e).
Hypothalamic Fto mRNA expression in fasted mice given i.p. glucose

Compared with the ad libitum-fed saline-treated controls in a second group of mice, body weight and blood levels of glucose, insulin and leptin were again significantly reduced in 30-h fasted mice (Figures 2a–d). Glucose treatment (i.p.) did not cause any significant changes in body weight, serum insulin and leptin levels in fasted mice (Figures 2a, c and d). Although there was a trend towards an increase in blood glucose levels by i.p. glucose injection, the effect did not reach statistical significance ($P=0.07$ by Tukey–Kramer test, Figure 2b). Blood glucose levels were not significantly different between saline-treated ad libitum-fed mice and glucose (i.p.)-treated fasted mice ($P=0.73$ by Tukey–Kramer test, Figure 2b). Fasting significantly reduced the hypothalamic levels of Fto mRNA by 53% compared with saline-treated ad libitum-fed mice (Figure 2e). Glucose treatment (i.p.) significantly increased Fto mRNA levels by 50% compared with i.p. saline injection in fasted mice (Figure 2e).

Hypothalamic Fto mRNA expression in fasted mice given i.c.v. glucose

Compared with the saline-treated control group, i.c.v. glucose treatment significantly increased the levels in blood glucose while having no effect on body weight, and serum insulin and leptin levels (Table 1). The i.c.v. injection of glucose significantly increased hypothalamic Fto mRNA levels compared with saline injection (Figure 3). The same glucose treatment did not cause any significant changes in Fto mRNA levels in the cortex ($P=0.41$ by Wilcoxon test, Figure 3).

Fto mRNA expression in hypothalamic tissues cultured ex vivo in the presence of low or high glucose

Exposure of hypothalamic explants to 10 mM glucose significantly increased Fto mRNA levels by 27% compared with 1 mM glucose (Figure 4). Fto mRNA levels in the cultured cortical explants were not significantly different between 1 and 10 mM glucose (Figure 4).

Hypothalamic Fto-expressing cells after fasting and i.p. glucose

By immunohistochemistry, Fto was found to be expressed throughout the brain, including the hypothalamic VMN, ARC, DMN, LPA, paraventricular nucleus and retrochiasmatic area. Quantitative analysis of Fto-positive neurons counterstained with DAPI showed Fto to be present in 72%, 62% and 72% of the DAPI-stained neurons in the VMN, ARC (Supplementary Figure 1) and DMN, respectively. Fasting for 30 h significantly reduced the number of Fto-immunoreactive cells in VMN, but not in ARC (Figures 5b and c). As in 30-h fasted mice taken for Fto mRNA analysis, blood glucose levels were significantly reduced in the fasted mice compared with the ad libitum-fed mice taken for immunohistochemical analysis (Figure 5a). The number of Fto-immunoreactive cells in both VMN and ARC of fasted mice was significantly increased after i.p. glucose treatment (Figures 5b and c). Blood glucose levels in fasted mice were significantly increased 2 h after i.p. glucose treatment compared with saline treatment (Figure 5a). There was a
A significant positive correlation between blood glucose levels and the number of VMN Fto-immunoreactive cells (Figure 5d). A positive correlation between blood glucose levels and the number of Fto-immunoreactive cells in ARC did not reach statistical significance ($r = 0.4697$, $P = 0.08$).

### Hypothalamic Fto expression after fasting and glucose treatment in obese mice

A 48 h fast significantly reduced blood glucose levels, and i.p. glucose treatment partially reversed this effect both in wild-type and ob/ob mice (Figure 6g). The 48 h fast caused a significant reduction in the number of Fto-immunoreactive cells in the VMN and LPA of wild-type mice, as well as in the ARC, in contrast to the absence of an effect in ARC after a 30-h fast period (Figures 6a, b and h–j). The 48 h fast did not alter the number of Fto-immunoreactive cells in any of these hypothalamic areas in ob/ob mice (Figures 6d, e and h–j). Glucose vs saline treatment of fasted wild-type mice significantly increased the number of Fto-immunoreactive cells in VMN and ARC by 13.1% and 31.8%, respectively (Figures 6b, c, h and i). This effect of glucose was absent in ob/ob mice (Figures 6e, f, h and i). The number of Fto-immunoreactive cells in LPA was not changed by i.p.

### Table 1

|                      | Saline | Glucose | $P^*$ |
|----------------------|--------|---------|-------|
| Body weight (g)      | 21.3 ± 0.6 | 21.6 ± 0.6 | 0.6864 |
| Blood glucose (mg/dl)| 64.3 ± 2.2 | 88.4 ± 4.2  | <0.0001 |
| Serum insulin (ng/ml)| 0.18 ± 0.04 | 0.16 ± 0.03  | 0.6205 |
| Serum leptin (ng/ml) | 0.76 ± 0.19 | 0.66 ± 0.23  | 0.7332 |

*$P$-values by Student's t-test or Wilcoxon test. Values are means ± s.e.m. ($n = 7–10$/group).

Figure 2  Fto mRNA expression in the hypothalamus in response to i.p. glucose treatment in mice. (a–d) Body weight, blood levels of glucose, insulin and leptin are lower in mice fasted for 30 h vs ad libitum-fed mice. This fasting effect is not reversed 1 h after i.p. glucose treatment. (e) Fto mRNA is reduced in hypothalamus after a 30-h fast and is increased after i.p. glucose treatment. mRNA expression measured by real-time PCR; values in saline-treated ad libitum-fed mice (fed/saline) were set to 100%. Data are means ± s.e.m. ($n = 6–8$/group). Groups that do not share a common letter are significantly different ($P$ < 0.05, Tukey-Kramer test or Wilcoxon test with Bonferroni correction).
glucose injection either in wild-type or ob/ob mice (Figure 6j). Neither fasting nor glucose injection affected the number of Fto-immunoreactive cells in DMN of wild-type or ob/ob mice (Supplementary Figure 2).

**Figure 3**  
**Fto mRNA expression in the hypothalamus and cerebral cortex in response to i.c.v. glucose treatment in mice.**  
*Fto* mRNA is reduced in the hypothalamus but not in the cerebral cortex after i.c.v. glucose treatment. Mice were fasted for 30 h, injected i.c.v. with saline or glucose every 6 h, and euthanized 1 h after the final injection. mRNA expression measured by real-time PCR; values in saline-treated mice were set to 100%. Data are means ± s.e.m. (*n* = 9–10/group). *P* < 0.05 compared with saline-treated group (Student’s *t*-test).

**Figure 4**  
**Fto mRNA expression in hypothalamic and cortical tissues cultured in low or high glucose medium.** The hypothalamus and cortex were excised from the mouse brain fed ad libitum and cultured *ex vivo* for 2 h in the presence of low (1 mM) or high (10 mM) glucose. mRNA expression measured by real-time PCR; values in low glucose treatment were set to 100%. Data are means ± s.e.m. (*n* = 6–7/group). *P*-values were obtained by Student’s *t*-test.

**Figure 5**  
**Number of Fto-immunoreactive cells in the hypothalamus in response to i.p. glucose treatment in mice.** (a) Blood glucose levels are reduced by a 30-h fast and increased 2 h after i.p. glucose treatment. (b–c) The number of Fto-immunoreactive cells in VMN is reduced after a 30-h fast and the number of those cells in VMN and ARC is increased 2 h after glucose treatment. (d) The number of VMN Fto-immunoreactive cells is positively correlated with blood glucose levels. Fto-immunoreactive cells were visualized by Immunohistochemistry, and the number of these cells was counted. Data are means ± s.e.m. (*n* = 5–8/group). Statistical analysis was performed using a one-way analysis of variance followed by Dunnett’s test (a–c).
Effect of glucose, 2-DG, and CCK-8 on food intake in wild-type and ob/ob mice

In wild-type mice, i.p. glucose or 2-DG injection significantly reduced or increased food intake compared with saline injection, respectively (Figures 7a and b). These effects were abolished in ob/ob mice (Figures 7a and b). I.p. injection of CCK-8 significantly reduced food intake both in wild-type and ob/ob mice (Figure 7c).
Discussion

Consistent with reports describing widespread Fto mRNA expression in the central nervous system, Fto-immunoreactive cells were found in many areas of the mouse brain, including various areas in the hypothalamus that are involved in the regulation of energy balance.6–9 In these hypothalamic areas, 60–70% of cells expressed Fto, as determined by immunolabeling for Fto, with DAPI staining for identification of cell nuclei. Based on the findings of a previous report demonstrating that Fto-expressing glial cells are very rare, Fto-immunoreactive cells detected in the present study are most likely neuronal cells.8 Localization of Fto in the nucleus is consistent with the proposed role of Fto in the regulation of gene expression as a DNA demethylase and transcriptional coactivator.6,27–29 These Fto expression patterns indicate that Fto has a role in the regulation of energy balance by altering the activities of subsets of hypothalamic neurons.

The association between FTO variants and obesity raises the possibility that Fto has a role in the regulation of metabolism and that expression of hypothalamic Fto may be regulated by nutritional signals. Consistent with this idea, fasting reduces hypothalamic Fto mRNA levels in mice with one exception in which 16 h fast increased hypothalamic Fto mRNA.6,7,9,10 We confirmed these findings by demonstrating that fasting is not only associated with reduced hypothalamic Fto mRNA levels, but also with a reduced number of Fto-immunoreactive cells in the mouse hypothalamus. The restriction of fasting-mediated alterations in Fto expression to specific hypothalamic areas,6,10 together with the lack of such alterations in the cerebral cortex, suggests that nutritional effects on Fto may occur within particular nutrient-sensing neuronal pathways in the hypothalamus. This further suggests the possibility that Fto may be part of hypothalamic neuronal pathways that monitor the metabolic status of the body.

In mice, the activity of hypothalamic Fto-expressing neurons was increased at the end of a meal, suggesting that these neurons may be responsive to feeding or increased availability of specific nutrients and hormones.9 Hypothalamic activity is altered by local glucose availability in the brain.23,30 Expression levels of hypothalamic nutrient-sensitive genes are altered by i.p. glucose injection, and this effect is blocked by i.c.v. injection of 2-DG.31 Furthermore, changes in glucose concentration result in alterations in nutrient-sensitive neuropeptide gene expression in hypothalamic tissues cultured ex vivo or in immortalized hypothalamic cell lines.32–34 In the present study, the reduced number of Fto-immunoreactive cells observed in the VMN and ARC after fasting was partially reversed by the elevation of blood glucose levels following i.p. glucose treatment. There was a significant positive correlation between blood glucose levels and the number of Fto-immunoreactive cells in VMN. We have also demonstrated that i.c.v. glucose administration increases hypothalamic Fto mRNA levels, and exposure of the hypothalamic explants to high glucose causes a significant increase in Fto mRNA levels. These data support the hypothesis that an increase in local glucose availability triggers an induction of Fto mRNA expression specifically in
hypothalamic nutrient-sensing neurons, and reduced glucose availability in the hypothalamus, at least partly, mediates the inhibitory effect of fasting on hypothalamic Fto expression. Fasting causes a variety of neuroendocrine and metabolic changes, such as a decline in leptin, insulin and glucose, and an elevation in glucocorticoids and free fatty acids. Changes in these nutritional and hormonal signals may contribute to the fasting-induced reduction in hypothalamic Fto expression and may also underlie the effects of feeding on Fto expression. For example, treatment with insulin, leptin or glucose reverses fasting-induced changes in hypothalamic nutrient-sensitive gene expression. Streptozotocin (STZ) treatment causes diabetes with hyperglycemia, hypoinsulinemia and hypoleptinemia in mice (Supplementary Figures 3a–c). Hypothalamic Fto mRNA levels were not different between control non-diabetic and STZ-induced diabetic mice (Supplementary Figure 3d). These data support the possibility that insulin and leptin also affect hypothalamic Fto expression. Thus, reduced insulin and leptin levels may counteract the stimulatory effect of glucose (hyperglycemia) on Fto expression, resulting in no alteration in hypothalamic Fto mRNA levels in STZ-induced diabetic mice. In contrast, in normal mice, we found that increased hypothalamic Fto mRNA levels after glucose treatment occurred in the absence of significant changes in serum insulin levels, suggesting that insulin may not be a major factor in regulating Fto expression under this condition.

The fasting-induced reduction and glucose-induced increase in hypothalamic Fto expression seen in wild-type mice were absent in ob/ob mice. A recent study also showed that long-term caloric restriction reduces hypothalamic Fto protein expression in wild-type mice and this response was absent in leptin-resistant db/db mice. These observations raise two possibilities. It may be that the leptin-deficient ob/ob mice are unresponsive to these metabolic cues due to either a direct or indirect role of leptin in regulating hypothalamic Fto expression. Alternatively, the high circulating glucose levels in ob/ob mice may have maximized hypothalamic Fto expression such that further i.p. glucose treatment was rendered ineffective. At present, we cannot distinguish between these two possibilities. It is noteworthy, however, that in contrast to the lack of an effect of fasting on hypothalamic Fto protein expression, fasting has been shown to reduce hypothalamic Fto mRNA in ob/ob mice. Hypothalamic Fto mRNA levels were reported to be reduced in ob/ob mice compared with those in wild-type mice and this effect was reversed by leptin treatment. Taken together, it appears that Fto expression is regulated at the transcriptional level in both leptin-dependent and leptin-independent manner, while at the translational levels hypothalamic Fto expression may be regulated by a leptin-independent mechanism. These findings also support the possibility that leptin as well as glucose participates in the regulation of hypothalamic Fto expression.

Although we show that glucose increases hypothalamic Fto expression, the mechanism behind this regulation is unknown. Leptin increases Fto expression via activation of the transcription factor cut-like homeobox 1 (CUX1) isoform P110 that is cleaved from the full-length CUX1 isoform P200 by a protease cathepsin L. Fasting reduces the activity of cathepsin L and the levels of P110 protein in the hypothalamus, suggesting the possibility that hypothalamic cathepsin L and CUX1 mediate the effect of metabolic signals including leptin on hypothalamic Fto expression. Interestingly, similar to the Fto knockout mice, cathepsin L-deficient mice exhibit the lean phenotype and improved glucose tolerance. Glucose and insulin affect the protein expression and activity of cathepsin L in several different cell types, suggesting that cathepsin L expression is regulated by nutrient and hormonal signals. These findings merit further studies to clarify the role of hypothalamic cathepsin L and CUX1 in the mediation of nutritional and hormonal regulation of hypothalamic Fto expression.

We hypothesized that dysregulation of hypothalamic Fto expression by nutrients may cause obesity. Hypothalamic glucose-sensing neurons are fewer in number and show abnormal responses to glucose in obese or obese-prone rats. The effect of glucose injection on hypothalamic activity was attenuated in obese humans compared with non-obese healthy individuals. Furthermore, glucose-induced feeding suppression is absent in obese animals. Both fasting and glucose treatment did not cause any significant changes in hypothalamic Fto expression in ob/ob mice in the present study. Although ob/ob mice showed reduced food intake in response to CCK, glucose, and 2-DG failed to cause significant changes in food intake in these mice. These data clearly indicate that the sensitivity of hypothalamic Fto-expressing neurons to nutrients, in particular glucose, is impaired in obesity, resulting in the absence of glucose-induced anorexia. High-fat diet feeding causes significant increases in weight gain and hypothalamic Fto mRNA expression without significant changes in energy intake, indicating that energy intake per gram of body weight is actually lower in high-fat diet-fed animals. These findings suggest that hypothalamic Fto expression is increased to protect against further weight gain by reducing energy intake in these animals. It is likely that certain nutritional factors such as glucose and fat have a stimulatory effect on Fto expression and hypothalamic Fto-expressing neurons function towards a counter-regulatory response against excessive energy intake and the subsequent development of obesity. Thus, the blunted response of hypothalamic Fto-expressing neurons to nutrient signals such as glucose may cause metabolic impairments. It is of interest to determine whether or not a similar impairment exists in diet-induced obese animals, which more closely mimic human obesity.

In conclusion, hypothalamic Fto gene and protein expression is regulated by metabolic signals, including glucose. Reduction in circulating glucose levels and/or hypothalamic
glucose availability at least partly mediates fasting-induced reduction in hypothalamic Fto expression. Obesity is associated with impairments in glucose-induced hypothalamic Fto expression and anorexia. Our findings support the hypothesis that hypothalamic Fto-expressing neurons have a role in the regulation of energy homeostasis by monitoring metabolic states of the body and enhancing Fto expression and/or activity in the hypothalamus is beneficial in reducing food intake and obesity.

Conflict of interest

The authors declare no conflict of interest.

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References

1. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 2007; 316: 889–894.
2. Fawcett KA, Barroso I. The genetics of obesity: FTO leads the way. Trends Genet 2010; 26: 266–274.
3. Fischer J, Koch L, Emmerling C, Vierkotten J, Peters T, Brüning JC et al. Inactivation of the Fto gene protects from obesity. Nature 2009; 458: 894–898.
4. Church C, Lee S, Bagga EA, McCaggart JS, Deacon R, Gerken T et al. A mouse model for the metabolic effects of the human fat mass and obesity associated FTO gene. PLoS Genet 2009; 5: e1000559.
5. Church C, Moir L, McMurray F, Girard C, Banks GT, Teboul J et al. Overexpression of Fto leads to increased food intake and results in obesity. Nat Genet 2010; 42: 1086–1092.
6. Gerken T, Girard CA, Tung YC, Weisberger CJ, Sandeek V, Hewitson KS et al. The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 2007; 318: 1469–1472.
7. Stratigopoulos G, Padilla SL, LeDuc CA, Watson E, Hattersley AT, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 2007; 316: 889–894.
8. Stratigopoulos G, Leduc CA, Cremona ML, Chung WK, Leibel RL. Cut-like homeobox 1 (CUX1) regulates expression of the fat mass and obesity-associated (FTO) and retinitis pigmentosa gtpase regulator interacting protein-1 like (RIGPIPL) genes, and co-ordinates leptin receptor signaling. J Biol Chem 2010; 286: 2155–2170.
9. Tung YC, Ayuso E, Shan X, Bosch F, O’Rahilly S, Coll AP et al. Hypothalamic-specific manipulation of Fto, the ortholog of the human obesity gene FTO, affects food intake in rats. PLoS One 2010; 5: e8771.
10. Berthoud HR, Mogenson GJ. Ingestive behavior after intracerebral and intracerebroventricular infusions of glucose and 2-deoxy-D-glucose. Am J Physiol 1977; 233: R127–R133.
11. Davis JD, Wirtshafter D, Asin KE, Brief D. Sustained intracerebroventricular infusion of brain fuels reduces body weight and food intake in rats. Science 1981; 212: 81–83.
12. Le Feuvre RA, Woods AJ, Stock MJ, Rothwell NJ. Effects of central injection of glucose on thermogenesis in normal, VMH-lesioned and genetically obese rats. Brain Res 1991; 547: 110–114.
13. Molina PE, Eltayeb K, Hourani H, Okamura K, Nannen LB, Williams P et al. Hormonal and metabolic effects of neuroglucopenia. Brain Res 1993; 618: 99–108.
14. Lam TK, Gutierrez-Juarez R, Pociak A, Bhanot S, Tso P, Schwartz GJ et al. Brain glucose metabolism controls the hepatic secretion of triacylglyceride-rich lipoproteins. Nat Med 2007; 13: 171–180.
15. Lam TK, Gutierrez-Juarez R, Pociak A, Rossetti L. Regulation of blood glucose by hypothalamic pyruvate metabolism. Science 2005; 309: 943–947.
16. Tsujii S, Bray GA. Effects of glucose, 2-deoxyglucose, phlorizin, and insulin on food intake of lean and fatty rats. Am J Physiol 1990; 258: E476–E481.
17. Song Z, Levin BE, McArdle JJ, Bakchos N, Routh V. Convergence of pre- and postsynaptic influences on glucosensing neurons in the ventromedial hypothalamic nucleus. Diabetes 2001; 50: 2673–2681.
18. Levin BE, Govek EE, Dunn-Meynell AA. Reduced glucose-induced neuronal activation in the hypothalamus of diet-induced obese rats. Brain Res 1998; 808: 317–319.
19. Matsuda M, Liu Y, Mahankali S, Pu Y, Mahankali A, Wang J et al. Altered hypothalamic function in response to glucose ingestion in obese humans. Diabetologia 1999; 42: 1033–1039.
20. Kim ER, Leckstrom A, Mizuno TM. Impaired anorectic effect of leptin in hypothalamic nuclei in diabetic mice. Brain Res 2008; 1204: 66–71.
21. Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B et al. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 2004; 428: 569–574.
22. Bergen HT, Monkman N, Mobbs CV. Injection with gold thioglucose impairs sensitivity to glucose: evidence that glucose-responsive neurons are important for long-term regulation of body weight. Brain Res 1996; 734: 332–336.
23. Poritsanos NJ, Wong D, Vrontakis ME, Mizuno TM. Regulation of hepatic PPARalpha2 and lipogenic gene expression by melatonin. Biochim Biophys Acta 2008; 1044: 384–388.
24. Poritsanos NJ, Lew PS, Mizuno TM. Relationship between blood glucose levels and hepatic Fto mRNA expression in mice. Biochem Biophys Acta 2010; 1800: 715–717.
25. Sanchez-Pulido L, Andrade-Navarro MA. The FTO (fat mass and obesity associated) gene codes for a novel member of the non-heme dioxygenase superfAMILY. BMC Biochem 2007; 8: 23.
26. Jia G, Yang CG, Yang S, Jian X, Yi C, Zhou L et al. Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO. FEBS Lett 2005; 582: 3313–3319.
27. Wu Q, Saunders RA, Szkudlarek-Mikho M, Serna Ide L, Chin KV. The obesity-associated Fto gene is a transcriptional coactivator. Biochem Biophys Res Commun 2010; 401: 390–395.
30 Dunn-Meynell AA, Govek E, Levin BE. Intracarotid glucose selectively increases Fos-like immunoreactivity in paraventricular, ventromedial and dorsomedial nuclei neurons. *Brain Res* 1997; 748: 100–106.

31 Wolfgang MJ, Cha SH, Sidhaye A, Chohnan S, Cline G, Shulman GI *et al.* Regulation of hypothalamic malonyl-CoA by central glucose and leptin. *Proc Natl Acad Sci USA* 2007; 104: 19285–19290.

32 Lee K, Li B, Xi X, Suh Y, Martin RJ. Role of neuronal energy status in the regulation of adenosine 5′-monophosphate-activated protein kinase, orexigenic neuropeptides expression, and feeding behavior. *Endocrinology* 2005; 146: 3–10.

33 Cai F, Gyulkhandanyan AV, Wheeler MB, Belsham DD. Glucose regulates AMP-activated protein kinase activity and gene expression in clonal, hypothalamic neurons expressing proopiomelanocortin: additive effects of leptin or insulin. *J Endocrinol* 2007; 192: 605–614.

34 Cheng H, Isoda F, Belsham DD, Mobbs CV. Inhibition of agouti-related peptide expression by glucose in a clonal hypothalamic neuronal cell line is mediated by glycolysis, not oxidative phosphorylation. *Endocrinology* 2008; 149: 703–710.

35 Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E *et al.* Role of leptin in the neuroendocrine response to fasting. *Nature* 1996; 382: 250–252.

36 Legradi G, Emerson CH, Ahima RS, Flier JS, Lechan RM. Leptin prevents fasting-induced suppression of prothyrotropin-releasing hormone messenger ribonucleic acid in neurons of the hypothalamic paraventricular nucleus. *Endocrinology* 1997; 138: 2569–2576.

37 Isse T, Ueta Y, Serino R, Noguchi J, Yamamoto Y, Nomura M *et al.* Effects of leptin on fasting-induced inhibition of neuronal nitric oxide synthase mRNA in the paraventricular and supraoptic nuclei of rats. *Brain Res* 1999; 846: 229–235.

38 Fekete C, Singru PS, Sanchez E, Sarkar S, Christofolite MA, Riberio RS *et al.* Differential effects of central leptin, insulin, or glucose administration during fasting on the hypothalamo-pituitary-thyroid axis and feeding-related neurons in the arcuate nucleus. *Endocrinology* 2006; 147: 520–529.

39 Schwartz MW, Sipols AJ, Marks JL, Sanacora G, White JD, Scheurink A *et al.* Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology* 1992; 130: 3608–3616.

40 Yang P, Yang FJ, Du H, Guan YF, Xu TY, Xu XW *et al.* Involvement of leptin receptor (LepRb)-STAT3 signaling pathway in brain FTO downregulation during energy restriction. *Mol Med* 2011; 17: 523–532.

41 Yang M, Zhang Y, Pan J, Sun J, Liu J, Libby P *et al.* Cathepsin L activity controls adipogenesis and glucose tolerance. *Nat Cell Biol* 2007; 9: 970–977.

42 Tournu C, Obled A, Roux MP, Deval C, Ferrara M, Bechet DM. Glucose controls cathepsin expression in Ras-transformed fibroblasts. *Arch Biochem Biophys* 1998; 360: 15–24.

43 Urbich C, Kernbach E, Rossig L, Zeiher AM, Dimmeler S. High glucose reduces cathepsin L activity and impairs invasion of circulating progenitor cells. *J Mol Cell Cardiol* 2008; 45: 429–436.

44 Huang X, Vaag A, Carlsson E, Hansson M, Ahren B, Groop L. Impaired cathepsin L gene expression in skeletal muscle is associated with type 2 diabetes. *Diabetes* 2003; 52: 2411–2418.

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