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ORIGINAL ARTICLE

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.
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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] Although 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.[6–10] 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

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nucleus (ARC) increased food intake and body weight, while enhanced Fto expression in ARC produced the opposite effect.[11] 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 glucose metabolism have a critical role in the regulation of food intake, energy expenditure, and carbohydrate and lipid metabolism.[12–17] 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.[18–21] 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/6J 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 CO_2 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.[22] 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.[23] Saline was used as a control vehicle instead of artificial cerebrospinal fluid (aCSF) because aCSF contains glucose.[22] 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). Mediobasal 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% CO_2. 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 (F0514SP, 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 for 3 h, and then transferred to 0.1 mol/l PBS containing 10% (w/v) sucrose and 0.04% (w/v) sodium azide 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 periarcuate 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 Dunnett'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).

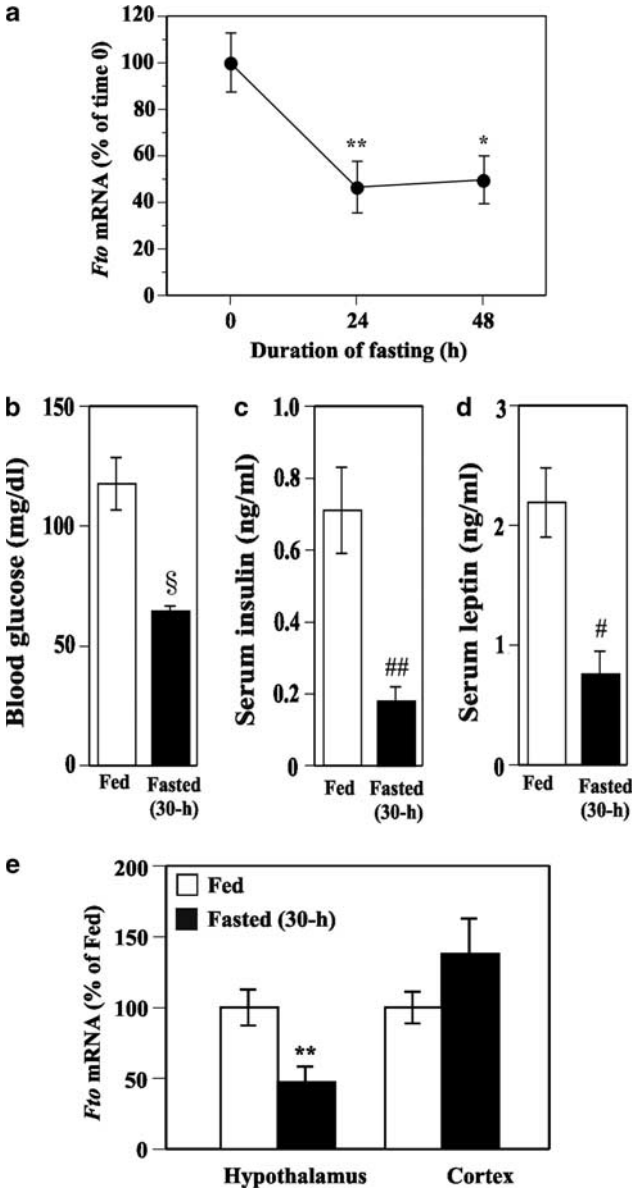

Figure 1 *Fto* mRNA expression in the hypothalamus and cerebral cortex in response to fasting. (**a**) Hypothalamic *Fto* mRNA is lower in mice fasted for 24 or 48 h vs *ad libitum*-fed mice (time 0). (**b**–**d**) Mice fasted for 30 h vs *ad libitum*-fed mice exhibit reduced blood levels of glucose, insulin and leptin. (**e**) *Fto* mRNA is reduced in hypothalamus but not cerebral cortex after a 30-h fast. mRNA expression measured by real-time PCR; values in *ad libitum*-fed mice were set to 100%. Data are means ± s.e.m. ($n = 7$–10/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.005$, $^{\#\#}P < 0.001$, $^{\S}P < 0.0005$ compared with *ad libitum*-fed group (Dunnett's test in (**a**) and Student's *t*-test in (**b**–**e**)).

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

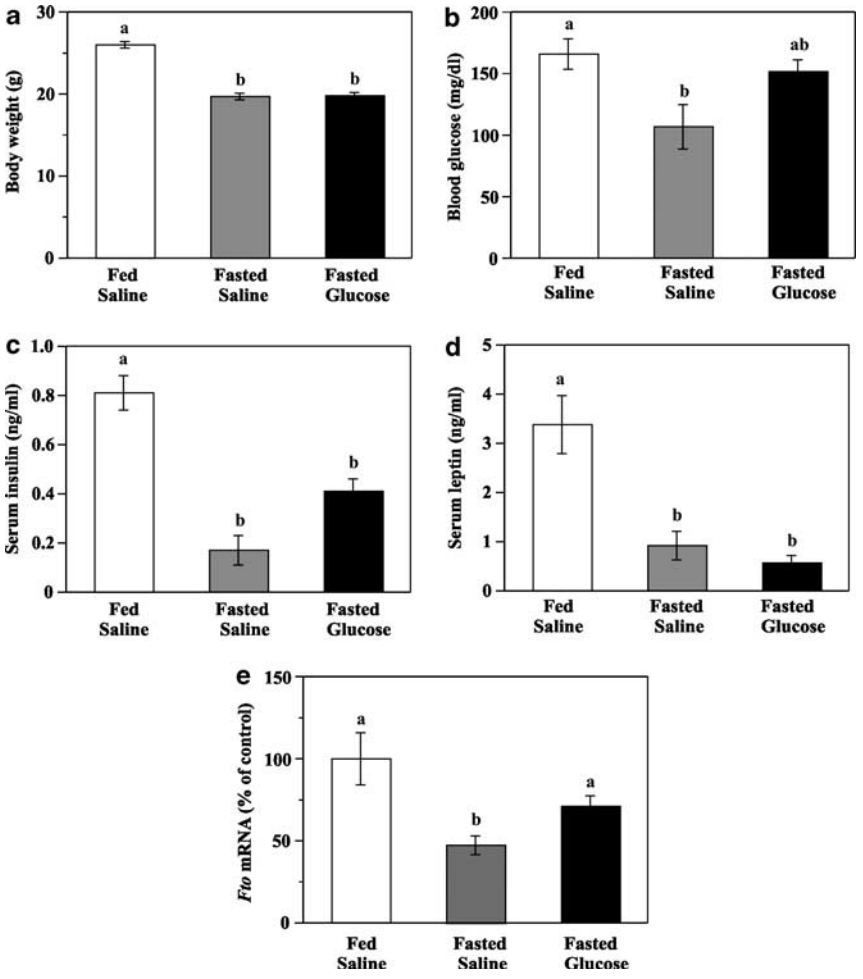

Figure 2 *Fto* mRNA expression in the hypothalamus in response to i.p. glucose treatment in mice. (**a**–**d**) Body weight, blood levels of glucose, insulin and leptin are lower in mice fasted for 30 h vs *ad libitum*-fed mice. This fasting effect is not reversed 1 h after i.p. glucose treatment. (**e**) *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).

Table 1 Effect of intracerebroventricular injection of glucose on body weight, concentrations of blood glucose, serum insulin and leptin in fasted mice

	Saline	*Glucose*	P[a]
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

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

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.

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).

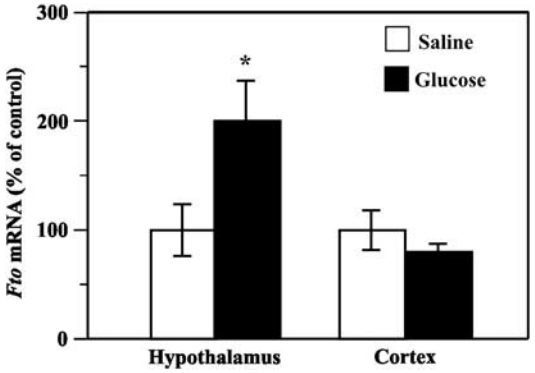

Figure 3 *Fto* mRNA expression in the hypothalamus and cerebral cortex in response to i.c.v. glucose treatment in mice. *Fto* mRNA is reduced in the hypothalamus but not in the cerebral cortex after i.c.v. glucose treatment. Mice were fasted for 30 h, injected i.c.v. with saline or glucose every 6 h, 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).

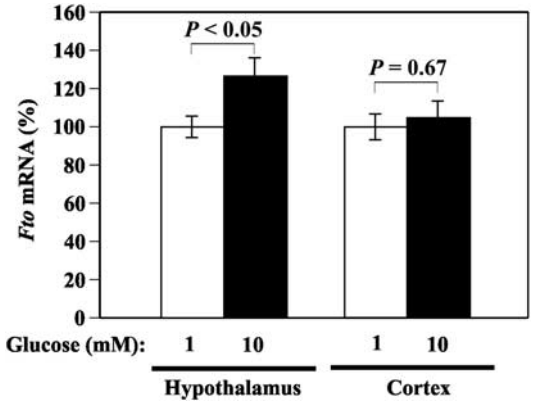

Figure 4 *Fto* mRNA expression in hypothalamic and cortical tissues cultured in low or high glucose medium. The hypothalamus and cortex were excised from the mouse brain fed *ad libitum* and cultured *ex vivo* for 2 h in the presence of low (1 mM) or high (10 mM) glucose. mRNA expression measured by 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.

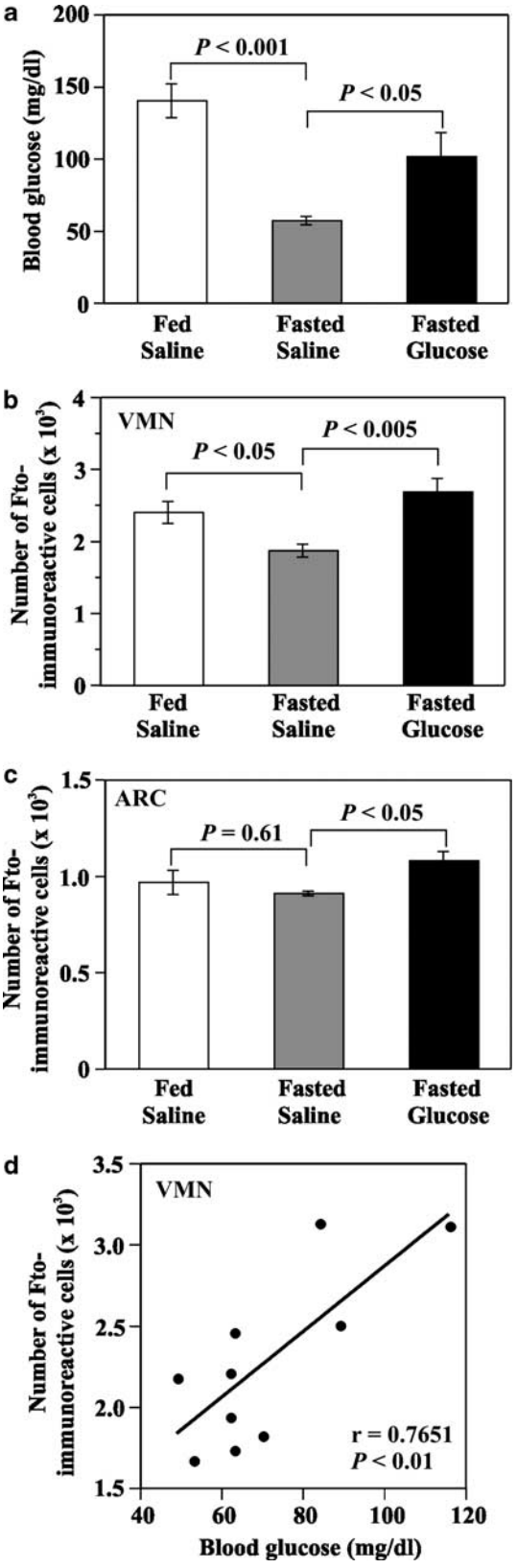

Figure 5 Number of Fto-immunoreactive cells in the hypothalamus in response to i.p. glucose treatment in mice. (**a**) Blood glucose levels are reduced by a 30-h fast and increased 2 h after i.p. glucose treatment. (**b**–**c**) The number of Fto-immunoreactive cells in VMN is reduced after a 30-h fast 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**).

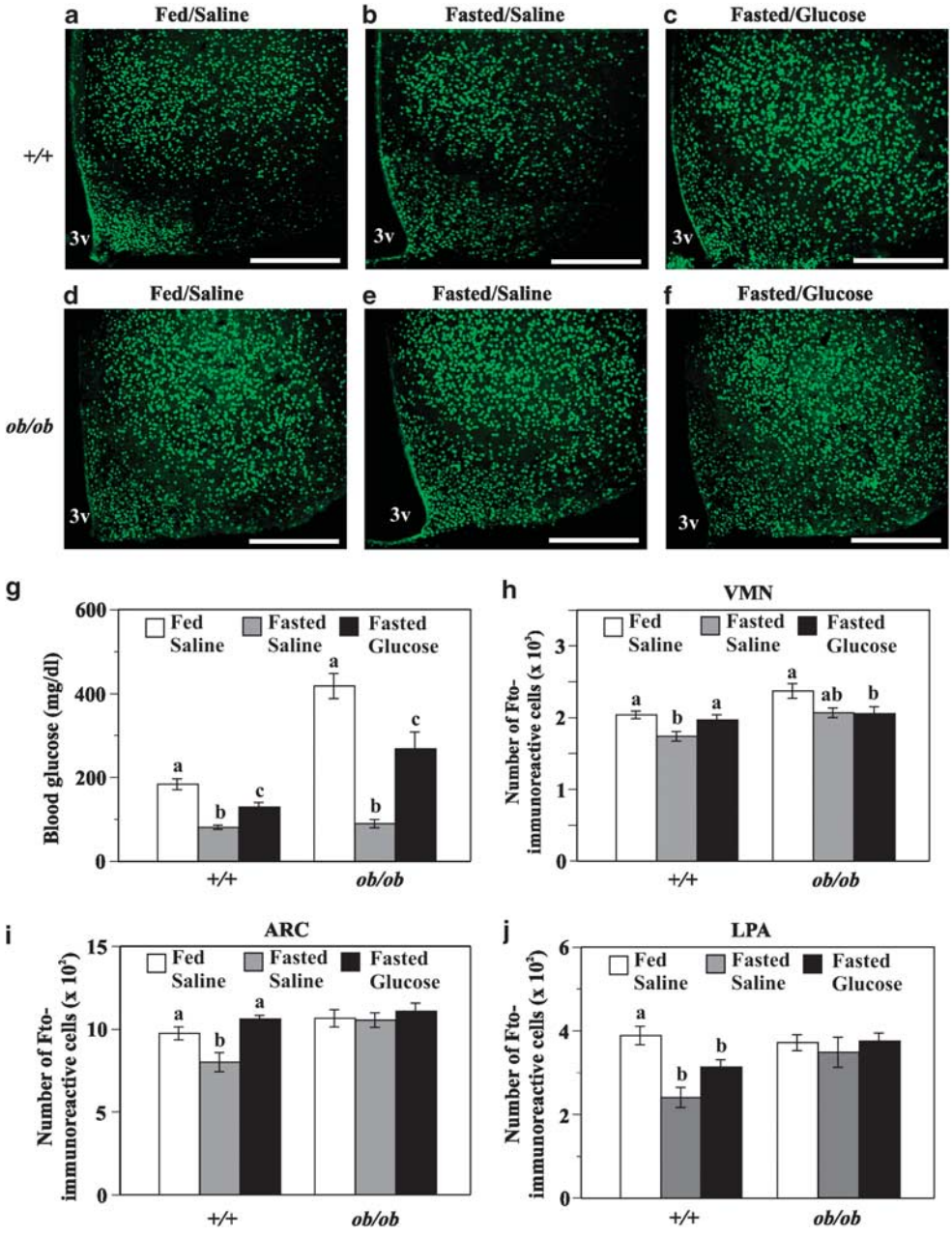

Figure 6 Number of Fto-immunoreactive cells in the hypothalamus in response to i.p. glucose treatment in obese mice. (**a**–**f**) Representative Fto immunofluorescent staining of hypothalamus of wild-type (+/+, **a**–**c**) and *ob/ob* (**d**–**f**) mice fed *ad libitum* and treated with i.p. saline (**a**, **d**), fasted for 48 h and treated with i.p. saline (**b**, **e**) or fasted for 48 h and treated with i.p. glucose (**c**, **f**). (**g**) Blood glucose levels are reduced by a 48-h fast and increased 2 h after i.p. glucose treatment. (**h**–**j**) The number of Fto-immunoreactive cells in VMN, ARC and LPA is reduced after a 48-h fast in wild-type mice, but not in *ob/ob* mice. Glucose treatment (i.p.) increases the number of Fto-immunoreactive cells in VMN and ARC in wild-type mice, but not in *ob/ob* mice. Fto-immunoreactive cells were visualized by Immunohistochemistry, and the number of these cells was counted. Data are means ± s.e.m. ($n = 7$–10/group). Groups that do not share a common letter are significantly different within each genotype ($P < 0.05$, Tukey–Kramer test). Scale bar = 500 μm. 3v: third ventricle.

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).

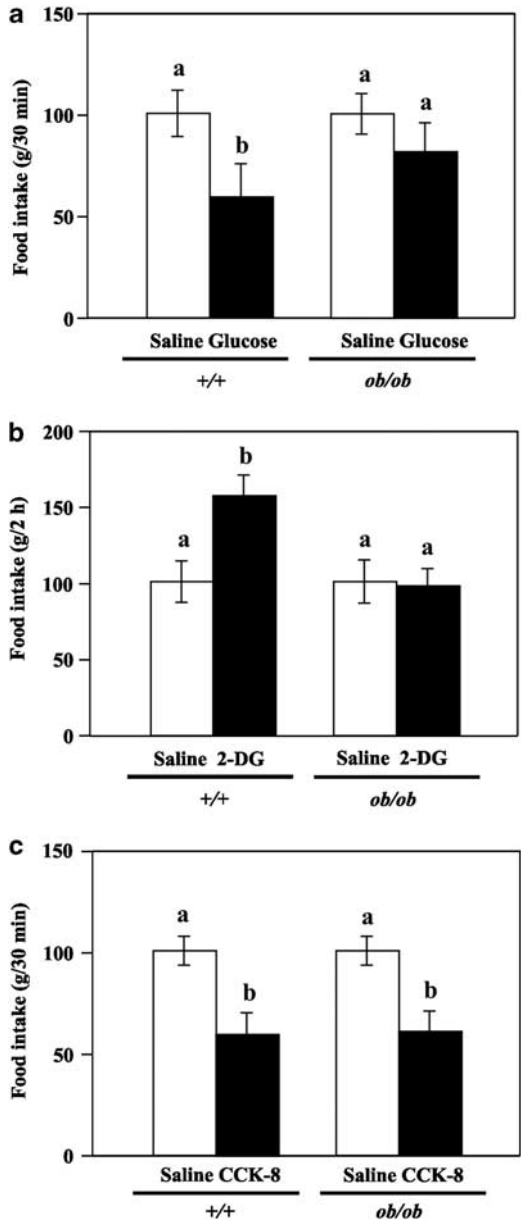

Figure 7 Feeding response to i.p. injection of glucose, 2-DG and CCK-8 in obese mice. Glucose-induced anorexia is absent in *ob/ob* mice (**a**). CCK-8 reduces food intake in both wild-type (+/+) and *ob/ob* mice (**b**). 2-DG increases food intake in wild-type mice, but not in *ob/ob* mice (**c**). Food intake was measured for 30 min (**a**, **b**) or 2 h (**c**) after i.p. injection of glucose or CCK-8, or 2-DG in mice fasted for 8 h (**a**, **b**) or fed *ad libitum* (**c**). Control mice received i.p. saline injection. Food intake in saline-treated control group in each genotype was set to 100%. Data are means ± s.e.m. ($n = 5$–6/group). Groups that do not share a common letter are significantly different ($P < 0.05$, Tukey–Kramer test).

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.[35–39] 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.[40] 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.[7] 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.[10] 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-dependent 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.[10] 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.[10] Interestingly, similar to the Fto knockout mice, cathepsin L-deficient mice exhibit the lean phenotype and improved glucose tolerance.[41] 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.[42–44] 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.[19,20] The effect of glucose injection on hypothalamic activity was attenuated in obese humans compared with non-obese healthy individuals.[21] Furthermore, glucose-induced feeding suppression is absent in obese animals.[18] 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.[11] 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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