Involvement of Leptin Receptor Long Isoform (LepRb)-STAT3 Signaling Pathway in Brain Fat Mass– and Obesity-Associated (FTO) Downregulation during Energy Restriction

Pei Wang,[1] Feng-Jiao Yang,[1*] Hui Du,[1] Yun-Feng Guan,[1] Tian-Ying Xu,[1] Xue-Wen Xu,[1] Ding-Feng Su,[1] and Chao-Yu Miao[1,2]*

[1]Department of Pharmacology, Second Military Medical University, Shanghai, China; and [2]Shanghai Key Laboratory of Vascular Biology at Ruijin Hospital and Shanghai Institute of Hypertension, Shanghai Jiaotong University School of Medicine, Shanghai, China

Obesity is an important risk factor for cardiovascular disease, diabetes and certain cancers. The fat mass– and obesity-associated (*FTO*) gene is tightly associated with the pathophysiology of obesity, whereas the exact role of *FTO* remains poorly understood. Here, we investigated the alternations of FTO mRNA and protein expression in the peripheral metabolic tissues and the brain upon energy restriction (ER) and explored the involvement of the leptin signaling pathway in FTO regulation under ER status. ER decreased the FTO mRNA and protein expression in hypothalamus and brainstem but not in periphery. Using double-immunofluorescence staining, FTO was found to be colocalized with the leptin receptor long isoform (LepRb) in arcuate nucleus of hypothalamus and the nucleus of the solitary tract. In LepRb mutant *db*/*db* mice, the FTO downregulation in brain and body weight reduction induced by ER were completely abolished. The enhanced phosphorylation of signal transducer and activator of transcription 3 (STAT3) induced by ER was also impaired in *db*/*db* mice. Moreover, leptin directly activated the STAT3 signaling pathway and downregulated FTO in *in vitro* arcuate nucleus of hypothalamus cultures and *in vivo* wild-type mice but not *db*/*db* mice. Thus, our results provide the first evidence that the LepRb-STAT3 signaling pathway is involved in the brain FTO downregulation during ER.

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INTRODUCTION

Obesity has reached epidemic proportions worldwide, and accumulating evidence indicates that obesity is a heterogeneous disorder affected by both genetic and nongenetic factors. However, few genes have been identified as robust and reproducible candidates for the genetic basis of obesity so far. Recently, the association between polymorphisms of the fat mass– and obesity-associated (*FTO*) gene and obesity was revealed by several large human genome-wide studies (1–3). This result was subsequently confirmed by numerous investigations (4–7). Moreover, it was reported that variation in the FTO locus may also affect weight loss in humans induced by lifestyle intervention, including reduction of dietary intake (8) and moderate-intensity exercise (9). Although many studies have examined the effect of *FTO* gene variation on obesity risk, the biological functions of FTO in obesity and the underlying mechanisms are largely unknown.

In rodents, it was reported that the FTO mRNA is widely expressed in many tissues, with especially high expression in the hypothalamus and pancreatic islets (10). Some reports have indicated that the *FTO* gene seems to exhibit its function in the central nervous system (CNS) by modulating energy homeostasis (10–12). Hypothalamic FTO mRNA expression was downregulated by fasting in mice (10,11) but was upregulated in rats (12). However, the exact influence of long-term energy restriction (ER) on FTO protein levels has never been determined. Furthermore, apart from the CNS mechanism, whether FTO in peripheral metabolic tissues is involved in the energy intake/expenditure and obesity is unclear.

It has been well established that leptin plays a critical role in feeding and energy storage (13–15). Leptin regulates energy balance throughout the body by controlling processes involved in energy intake and utilization both in the periphery and the brain (13,16). Leptin acts through its receptor to regulate the downstream signaling pathways. Alternative splicing of the transcript from a single leptin receptor gene produces multiple leptin recep-

**PW and F-JY contributed equally to this work.*

Address correspondence and reprint requests to *Chao-Yu Miao or Ding-Feng Su, Department of Pharmacology, Second Military Medical University, 325 Guo He Road, Shanghai 200433, China. Phone: +86-21-81871271; Fax: +86-21-65493951; E-mail (C-YM): cymiao@smmu.edu.cn; E-mail (D-FS): dfsu2008@gmail.com.*

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tor isoforms, of which the long isoform (LepRb) is the major signaling form of the leptin receptor (17), especially in brain (13). ER is a common and robust intervention used to reduce body weight in obese patients. Moreover, it was reported that ER can enhance leptin sensitivity by enhancing both LepRb expression and signaling capacity in the brain to prevent central leptin resistance (18,19). Given that ER is one of most effective methods for decreasing body weight and FTO is thought to be involved in the pathophysiological process of obesity, we therefore consider the clarification of the association of the leptin signaling pathway and FTO valuable. Although serum leptin levels were reported to be associated with variants of the *FTO* gene (20–22), the association between FTO and the leptin signaling pathway is largely unknown. Thus, we tested whether ER for 8 or 50 weeks can alter FTO expression in the periphery and CNS. After finding the modulation of FTO was limited in the brain, we hypothesized that the CNS LepRb may be of important physiological significance in modulating FTO expression upon ER.

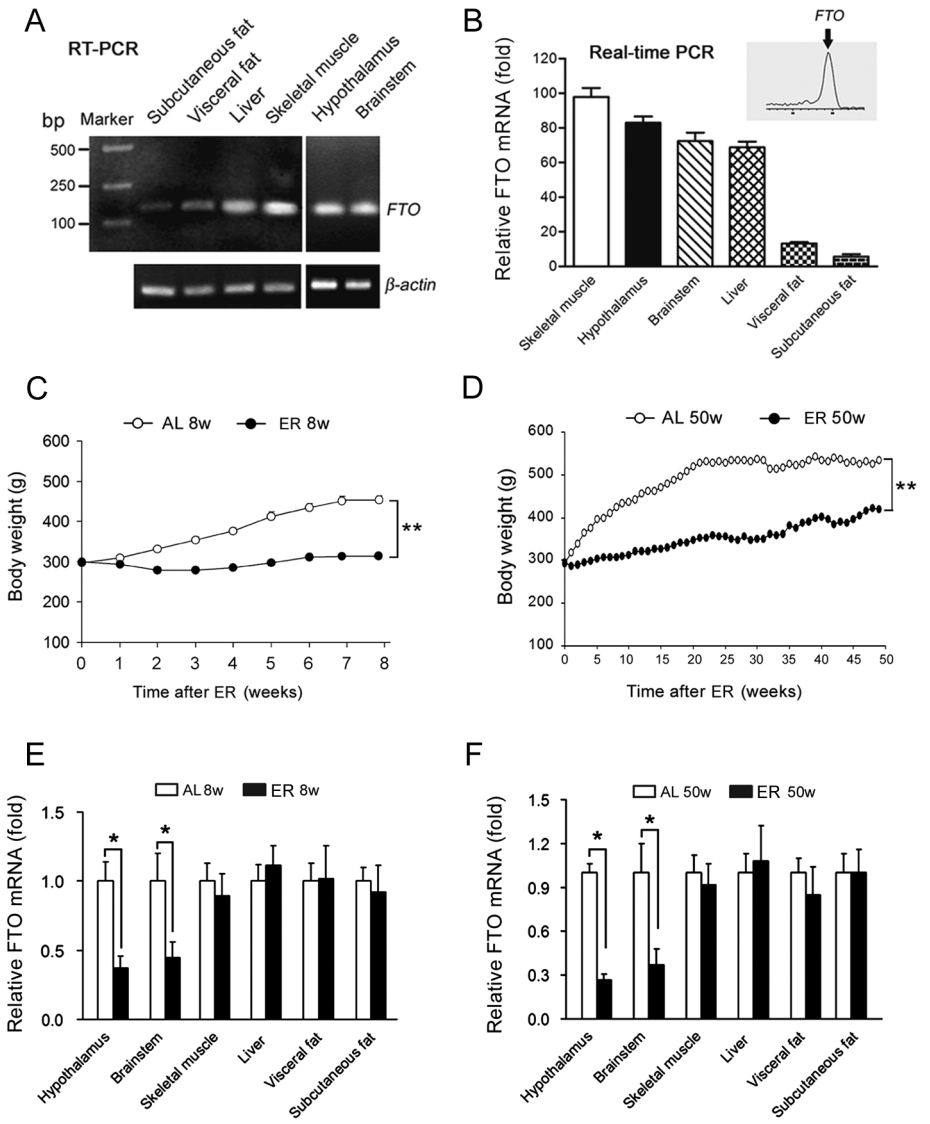

Figure 1. FTO mRNA levels in rat tissue and ER downregulate FTO mRNA levels in hypothalamus and brainstem but not in peripheral metabolic tissues. (A and B) RT-PCR (n = 4) or real-time PCR (n = 4) showing the FTO mRNA levels in skeletal muscle, liver, hypothalamus, brainstem and adipose tissues. (C and D) Body weight curves of rats. n = 8 per group in (C) and n = 4 per group in (D). $^{**}P < 0.01$ versus AL. (E) Changes of FTO expression induced by 8 wks of ER. (F) Changes of FTO expression induced by 50 wks of ER. n = 8 per group in (E) and n = 4 per group in (F). $^{*}P < 0.05$ versus AL. 8w, 8 weeks; 50w, 50 weeks.

MATERIALS AND METHODS

Animals and Reagents

The animal studies were performed in accordance with the *Guide for Care and Use of Laboratory Animals* published by the National Academy Press (1996). Eight-week-old male Sprague-Dawley rats were purchased from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). Ten-week-old male BKS.Cg-Dock7^{m} +/+ *Lepr*^{db}/J mice (BKS-*db*/*db*, The Jackson Laboratory stock number 000642) and their lean C57BLKS/J wild-type (WT, The Jackson Laboratory stock number 000662) controls were obtained from the National Model Animal Research Center (Nanjing, China). Animals were housed in controlled conditions (temperature 23 ± 2°C, humidity 60 ± 10%, and lighting from 8:00 AM to 8:00 PM), with free access to water.

FTO polyclonal antibody, LepRb goat polyclonal antibody (SC-1832, recommended for detection of long form of leptin receptor long isoform [LepRb]) and signal transducer and activator of transcription 3 (STAT3) inhibitor S3I-201 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Another LepRb chicken polyclonal affinity purified antibody (catalog number CH14104) was from Neuromics (Edina, MN, USA). Recombinant leptin was purchased from Phoenix Pharmaceuticals Inc. (Phoenix, AZ, USA). The FTO (clone number H-300, SC-98768) is a rabbit polyclonal antibody raised against amino acids 252–505 mapping at the C-terminus of FTO of human origin. Because these amino acids were identical among human, rat and mouse, this antibody

could be used to detect FTO protein from the three species. The specificity of this antibody was tested in our lab (data not shown) and by the manufacturer (http://www.scbt.com/picts.php?pict_id = 162218). Anti–phospho-STAT3^{Tyr705}, anti–total-STAT3, anti–phospho-Jak2$^{Tyr1007/1008}$ and anti–total-JAK2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti–phospho^{Ser9}-GSK3, anti–total-GSK3 and JAK2 inhibitor AG-490 were purchased from Calbiochem (San Diego, CA, USA). Anti–β-actin and GSK-3 inhibitor L803-mts were purchased from Sigma (St. Louis, MO, USA). IRDye 700CW- or 800CW-conjugated secondary antibodies were obtained from Li-Cor Bioscience (Lincoln, NE, USA). Dylight 488–conjugated goat antirabbit and Cy3-conjugated goat antimouse IgG were purchased from Jackson ImmunoResearch Inc. (West Grove, CA, USA).

Research Design

After 1 wk of acclimation, rats were divided into two groups. The animals in the energy restriction group (ER group) had access to 60% of the *ad libitum* consumption of the control animals (*ad libitum* [AL] group). The standard chow for rats or mice was purchased from Sino-British SIPPR/BK Lab Animal Ltd. This chow diet contained a variety of nutrient substances including 20% protein, 5% fat and other essential vitamins/micro-elements. The amount of food intake of AL animals was measured every week to adjust the amount of ER animal food intake weekly. Body weights of rats were recorded every week. After 8 and 50 wks of ER, rats were anesthetized with pentobarbital (40 mg/kg) and blood was swiftly collected. The mesenteric adipose was considered to be visceral adipose. Hypothalamus and brainstem were dissected according to the method described by Glowinski and Iversen (23) and Andrews and Murphy (24). Tissues were harvested as described previously (25). Serum glucose levels were measured as described previously (26). Serum insulin levels were determined by an enzyme-linked immunosorbent assay kit (Linco Research, St. Louis, MO, USA).

After 1 wk of accommodation, WT and *db/db* mice were divided into four groups: WT-AL, WT-ER, *db/db*-AL and *db/db*-ER groups. Given that the food intakes of WT mice were not the same as those of *db/db* mice, WT-ER mice were subjected to ER with 60% of food compared with WT-AL mice, and *db/db*-ER mice were subjected to ER with 60% of food compared with *db/db*-AL mice, respectively. Mice body weights were recorded every week. After 8 wks of ER, mice were anesthetized with pentobarbital (40 mg/kg), and the hypothalamus and brainstem were harvested.

Reverse Transcript Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction

Total RNA was extracted from tissues using Trizol (Invitrogen, Carlsbad, CA, USA) as described previously (27), and 1 μg RNA was reverse-transcribed to cDNA using RNA reverse transcriptase (Promega, Madison, WI, USA) (28). Polymerase chain reaction (PCR) products were electrophoretically separated with 1.2% agarose gels. Real-time PCR was performed using an Opticon Monitor 3 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and SYBR Premix Ex Taq Mixture (Takara, Otsu, Japan) as described previously (29). The following primers were used: FTO (for real-time PCR), forward: 5′-GCCGCATGTC AGACCTTCC-3′, reverse: 5′-GACCT GTCCACCAAGTTCTCG-3′; FTO (for reverse transcript [RT]-PCR), forward: 5′-TTTGCACTGATTGGTGGTGTG-3′, reverse: 5′-GGACGGCAGACAGAA TTTCACT-3′; β-actin, forward: 5′-AGACC TCTATGCCAACACAGTGC-3′, reverse: 5′-GAGCCACCAATCCACACAGAGT-3′. β-Actin was used as internal control, and the amount of RNA was calculated by the comparative threshold cycle method as recommended by the manufacturer. The real-time PCRs were performed with transcription efficiency >90%. All the data were calculated from duplicate reactions.

Western Blotting

Western blotting analysis was performed as described in our previous reports (30). Tissues were homogenized by protein extraction reagent (Beyotime, Nanjing, China) supplemented with protease inhibitor cocktail (Pierce, Rockford, IL, USA) and phosphatase inhibitor cocktail (Pierce). About 20 μg of samples were run on 10% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The proteins were elec-

Table 1. Characteristics of AL and ER rats.

	AL 8 wks (n = 8)	ER 8 wks (n = 8)	AL 50 wks (n = 4)	ER 50 wks (n = 4)
Body weight (g)	455 ± 11	325 ± 4[a]	535 ± 32	412 ± 26[b]
Left anterior gastrocnemius muscle (g)	1.08 ± 0.04	1.00 ± 0.03	1.42 ± 0.10	1.02 ± 0.04[b]
Liver (g)	15.99 ± 0.72	9.53 ± 0.22[a]	11.87 ± 0.72	9.37 ± 0.22[c]
Subcutaneous fat (g)	18.65 ± 0.73	10.16 ± 0.60[a]	20.32 ± 2.19	9.57 ± 0.89[c]
Visceral fat (g)	19.35 ± 1.20	6.85 ± 0.18[a]	21.90 ± 2.12	14.55 ± 0.79[b]
Epididymal fat (g)	6.12 ± 0.44	2.59 ± 0.15[a]	7.59 ± 0.70	5.67 ± 0.44[b]
Retroperitoneal fat (g)	6.55 ± 0.68	1.59 ± 0.20[a]	8.32 ± 1.18	4.34 ± 0.36[b]
Perirenal fat (g)	2.36 ± 0.20	0.75 ± 0.05[a]	1.81 ± 0.04	1.68 ± 0.16
Mesenteric fat (g)	4.32 ± 0.37	1.92 ± 0.08[a]	4.18 ± 0.35	2.85 ± 0.05[c]
Serum glucose (mmol/L)	7.26 ± 0.45	8.08 ± 0.33	7.48 ± 0.28	7.65 ± 0.60
Serum insulin (mmol/L)	0.55 ± 0.05	0.26 ± 0.06[a]	0.47 ± 0.04	0.29 ± 0.02[b]

[a] $P < 0.01$, ER 8 wks versus AL 8 wks.
[b] $P < 0.05$, ER 50 wks versus AL 50 wks.
[c] $P < 0.01$, ER 50 wks versus AL 50 wks.

trotransferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with primary antibody for 2–3 h at 25°C and then incubated with corresponding IRdye-800CW– or 700CW-conjugated secondary antibodies. The images were captured and analyzed by the Odyssey infrared fluorescence imaging system (Li-Cor Bioscience). The adoption of this system allowed for superior sensitivity, linearity and reproducible quantitation compared with formerly used X-ray film–based technologies for Western blotting (31). Each experiment was repeated at least three times.

Immunofluorescence

Immunofluorescence analysis was performed as described previously with some modification (29). Frozen 20-μm-thick rat brain sections were fixed in 4% paraformaldehyde, blocked by 8% normal goat serum and incubated in specific primary antibodies (FTO, rabbit polyclonal antibody, 1:100 dilution; LepRb, mouse monoclonal antibody, 1:100 dilution) at 4°C overnight. After being washed three times with phosphate-buffered saline, the sections were incubated with Dylight 488–conjugated goat antirabbit or Cy3-conjugated goat antimouse IgG. Normal goat IgG was used for the negative control. No visual fluorescence was observed in the negative control. The images were recorded using a confocal laser scanning microscope (Olympus, FV1000, Tokyo, Japan) and analyzed using Image Pro Plus software.

Isolated Arcuate Nucleus of Hypothalamus Explant Cultures

Isolated arcuate nucleus of hypothalamus (ARH) explant cultures were prepared according to a previous report with some modifications (32). The hypothalamuses were collected under anesthesia (sodium pentobarbital, 40 mg/kg i.p.) from postnatal day 7 WT mice and then sectioned at 200~300 μm. The ARH was carefully dissected under a stereomicroscope. Explants were cultured onto poly-L-lysine precoated glass coverslips and incubated in serum-free medium (33). The EOL-1 medium containing either recombinant leptin (100 ng/mL) or vehicle was replaced every day. After 1 or 3 d *in vitro*, the explants were washed with ice-cold phosphate-buffered saline and then lysed for Western blotting analysis.

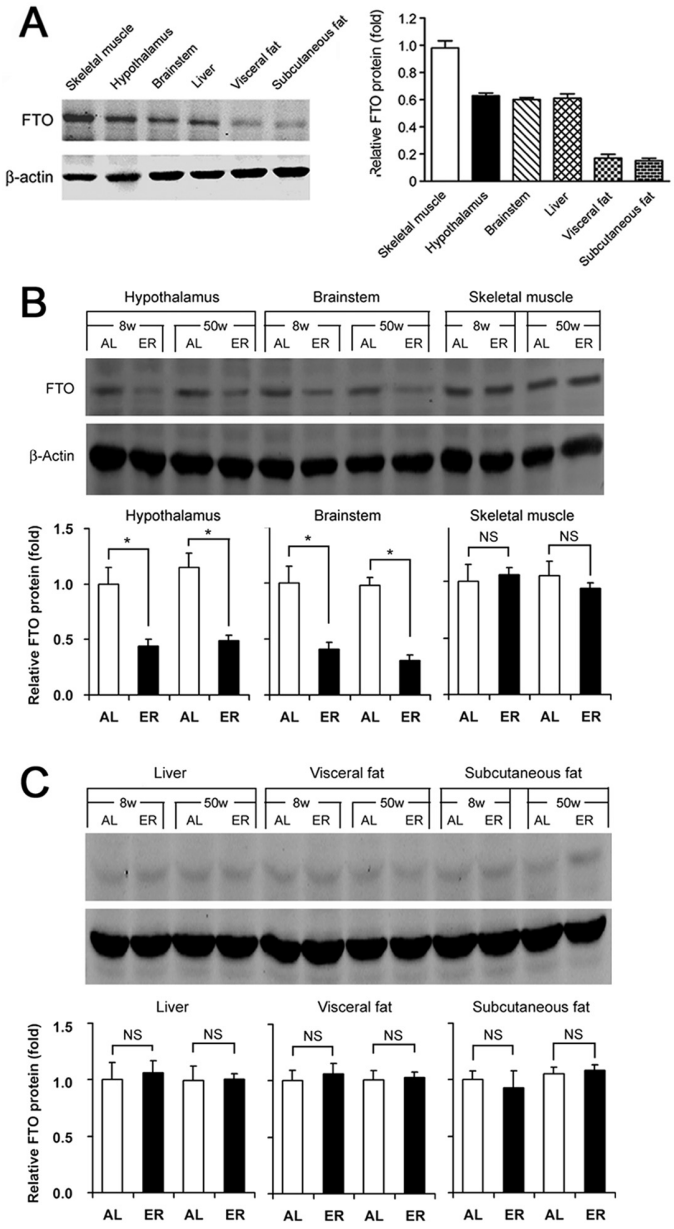

Figure 2. FTO protein levels in rat tissues and effects of ER on the FTO protein expression. (A) Representative image of FTO protein expressions in rat tissues and quantitative analysis. (B) ER downregulates FTO protein expression in hypothalamus and brainstem, but not in skeletal muscle. n = 4 per group. (C) ER does alter FTO protein expression in skeletal muscle, liver and adipose tissues. NS, no significance. *$P < 0.05$ versus AL.

Intracerebroventricular Injection of Leptin

Intracerebroventricular injection of leptin was performed as described previously with some modifications (34). The coordinates for lateral ventricular injection were as follows: anterior–posterior (from bregma): –0.5 mm; dorsal–ventral

(from skull surface): –2.5 mm; and medial–lateral: 1 mm. A single-bolus leptin (1 μg, 1 μg/μL) was injected into the lateral ventricle. Four hours later, rats were killed and the hypothalamuses were harvested for immunoblotting.

Statistical Analysis

Data are represented as means ± SD. Differences were evaluated by two-tailed Student *t* test (two groups) or one-way analysis of variance followed by Tukey *post hoc* test (three or more groups). Statistical significance was set at $P < 0.05$.

RESULTS

FTO mRNA Expression in Tissues

Initially, we used two different methods, RT-PCR and quantitative real-time PCR, to evaluate the mRNA expression of FTO in several metabolic-sensitive tissues both in the periphery and CNS. As shown in Figure 1A and B, among the peripheral metabolic-sensitive tissues, the FTO mRNA expression in liver and skeletal muscle was higher than that in adipose tissues. Moreover, the mRNA expression of FTO in hypothalamus and brainstem was high. Interestingly, FTO mRNA expression in skeletal muscle was even higher than that in hypothalamus and brainstem.

ER Downregulates FTO mRNA Expression in Brain Rather Than in Peripheral Tissues

Effects of ER were determined at two time points. ER rats had a lower body weight and fat pad weights. Additionally, as expected, the serum insulin levels of both kinds of ER rats were lower than those of AL rats, whereas serum glucose did not change (Table 1). Figure 1C and D show the body weight curves of AL and ER (8 and 50 wks) rats. We determined the effect of ER on FTO mRNA expression in tissues using real-time PCR analysis. As shown in Figure 1E and F, FTO mRNA levels were significantly downregulated in hypothalamus (8 wks: 37% compared with AL, $P < 0.05$; 50 wks: 28% compared with AL, $P < 0.05$) and brainstem (8 wks: 45% compared with AL, $P < 0.05$; 50 wks: 37% compared with AL, $P < 0.05$). However, we did not detect any alternations of FTO mRNA expression in liver, skeletal muscle and adipose of ER rats (see Figure 1E, F).

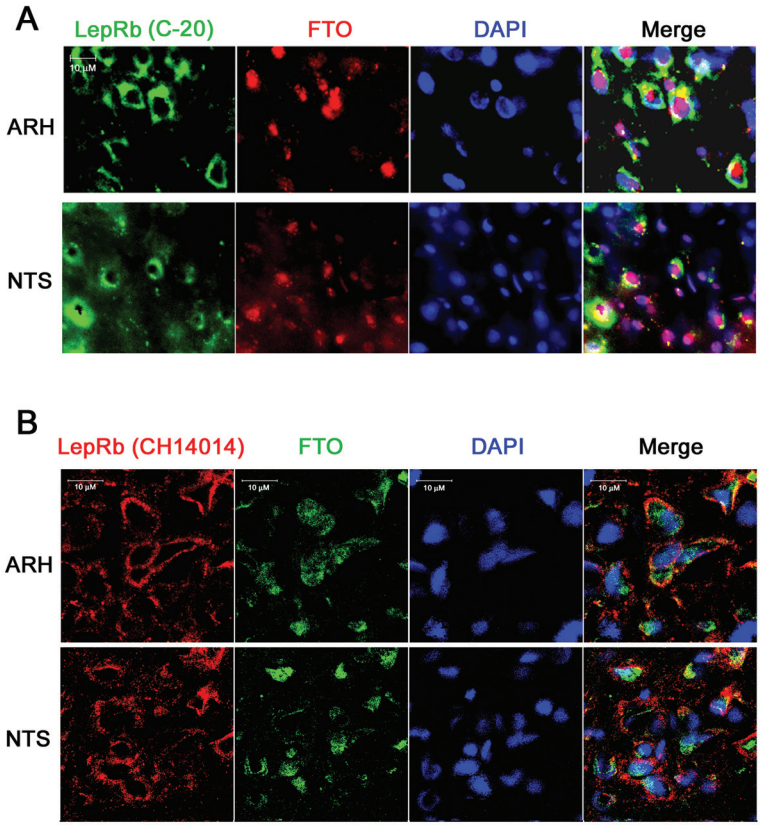

Figure 3. Analysis of colocalization of FTO and LepRb. Immunofluorescence staining showing the FTO colocalizes with LepRb in neural cells of rat hypothalamus and brainstem. Two different kinds of LepRb antibodies were used. (A) Frozen brain sections were incubated with LepRb (clone number C-20; goat antirat) and FTO (rabbit antirat) antibodies and then incubated with Dylight 488–conjugated secondary antibody (donkey antigoat, *green*) or Cy3-conjugated secondary (donkey antirabbit, *red*). (B) Frozen brain sections were incubated with LepRb (clone number CH14014; chicken antirat) and FTO (rabbit antirat) antibodies and then incubated with Cy3-conjugated secondary antibody (goat antichicken, *red*) or FITC-conjugated secondary (goat antirabbit, *red*). Nuclei were stained by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). NTS, nucleus of the solitary tract.

FTO Protein Expression in Brain Is Downregulated by ER

Because mRNA levels do not always indicate the protein levels, we studied the FTO protein expression using infrared fluorescence Western blotting analysis to evaluate whether ER can modulate FTO protein expression accordingly. First, the patterns of FTO protein expression in various rat tissues were determined. Similar to mRNA expression, we observed high expression of FTO in skeletal muscle, liver, hypothalamus and brainstem and low expression in adipose tissues (Figure 2A). In hypothalamus, 8 and 50 wks of ER induced significant downregulation of FTO protein expression, respectively ($P < 0.05$, Figure 2B). In brainstem, similar changes were also observed (see Figure 2B). However, consistent with the results of mRNA expression, ER did not alter the protein expression of FTO in skeletal muscle (see Figure 2B), liver (Figure 2C) and adipose tissues (see Figure 2C).

Colocalization of FTO and LepRb in Hypothalamus and Brainstem

Because ER, a nutritional intervention, only modulated FTO expression in brain, we tried to explore how ER can affect FTO expression. We investigated the involvement of the leptin signaling pathway in the modulation of FTO expression induced by ER. Double immunofluorescence staining was performed to investigate the distribution of FTO and LepRb in the rat hypothalamus and brainstem. Here, we used two clones of antibodies against LepRb. One was goat antirat polyclonal antibody (clone number C-20, Figure 3A), and another was chicken antirat polyclonal antibody (clone number CH14014, Figure 3B). LepRb colocalized with FTO in the ARH and the nucleus of the solitary tract (see Figure 3A, B).

Reduction of Body Weight and Downregulation of Brain FTO Induced by ER Are Impaired in *db/db* Mice

To further investigate whether ER modulates FTO expression via the leptin signaling pathway, we subjected *db/db* mice with LepRb mutation and dysfunction to ER. Body weight was recorded weekly. In WT mice, 8 wks of ER induced weight loss (Figure 4A), whereas this effect was totally abolished in *db/db* mice (Figure 4B). We determined the FTO expression in *db/db* mice and their WT control mice. As shown in Figure 4C and D, there were no significant differences of FTO expression between WT and *db/db* mice in normal status (both in hypothalamus and brainstem). Consistent with the results obtained in rats, after 8 wks of ER treatment, WT-ER mice displayed decreased FTO expression in hypothalamus and brainstem compared with WT-AL mice (Figure 4EF). In contrast, FTO was not downregulated in hypothalamus and brainstem in *db/db*-ER mice compared with *db/db*-AL mice (see Figure 4E, F). This result indicates that dysfunction of LepRb abolishes the effects of ER on FTO expression in brain.

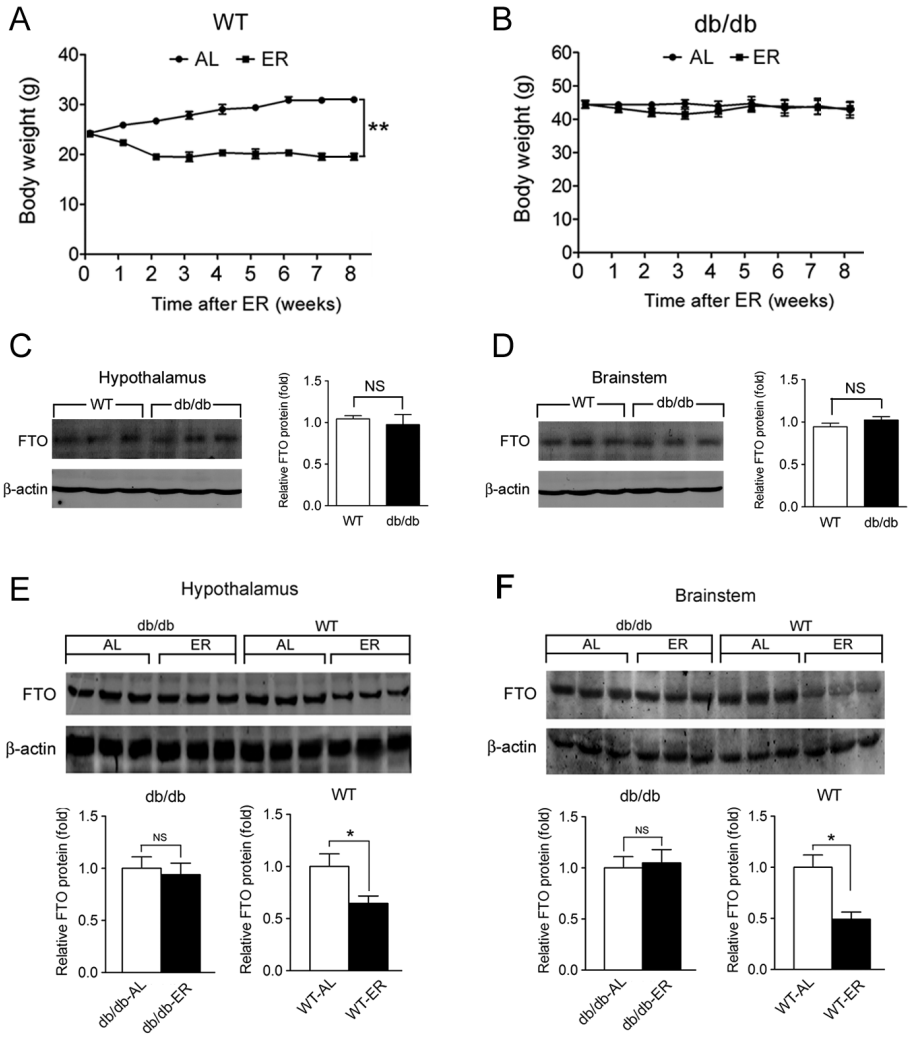

Figure 4. Effects of ER on body weight and FTO protein expression in WT and *db/db* mice. (A, B) Curves of body weight of WT and *db/db* mice. n = 6 per group. (C, D) FTO protein expression in WT and *db/db* mice in normal status. n = 3 per group. (E, F) After 8 wks of ER, FTO protein expression in hypothalamus and brainstem was decreased in WT mice but not in *db/db* mice. The 20-μg proteins were loaded. n = 3 per group. NS, no significance. **P* < 0.05 versus AL.

Activation of STAT3 Signaling by ER in Brain Is Impaired in *db/db* Mice

Because FTO did not directly interact with LepRb, we investigated whether LepRb-related signaling pathways were involved in the effects of ER on FTO expression *in vivo*. We determined the STAT3, GSK3 and JAK2 pathways under ER stimulation in hypothalamus (Figure 5A) and brainstem (Figure 5B) of WT and *db/db* mice. ER significantly activated the STAT3 pathway in hypothalamus and brainstem in WT mice rather than in *db/db* mice. In contrast, ER inhibited activation of the GSK3 pathway in both WT and *db/db* mice, indicating that dysfunction of LepRb did not affect the ER effect on the GSK3 pathway. Moreover, ER did not affect the JAK2 signaling pathway in both WT and *db/db* mice in this study.

Leptin Downregulates FTO in Isolated ARH Cultures via STAT3 but Not the GSK-3 or JAK2 Signaling Pathway

To provide direct evidence for the regulation of leptin on FTO expression, iso-

lated ARH cultures were used. Leptin incubation (100 ng/mL, 1 and 3 d) significantly decreased FTO protein expression (Figure 6A). Additionally, this observation was totally abolished by the STAT3-specific inhibitor S3I-201, but not by the GSK-3 inhibitor L803-mts or JAK2 inhibitor AG-490 (Figure 6B).

Leptin Downregulates FTO and Activates the STAT3 Signaling Pathway *In Vivo*

A single bolus leptin injection into lateral ventricle decreased hypothalamic FTO expression in WT mice but not in *db/db* mice (Figure 6C). Intracerebroventricular injection of leptin also activated phosphorylation of STAT3 in WT mice but not in *db/db* mice (Figure 6D). Moreover, we observed a significant increase of phosphorylation of STAT3 in WT-ER mice compared with WT-AL mice (see Figure 6D). Interestingly, leptin further increased p-STAT3 levels in WT-ER mice (see Figure 6D). Meanwhile, FTO was downregulated by ER and leptin (see Figure 6D). However, all these changes were not observed in *db/db* mice (see Figure 6D).

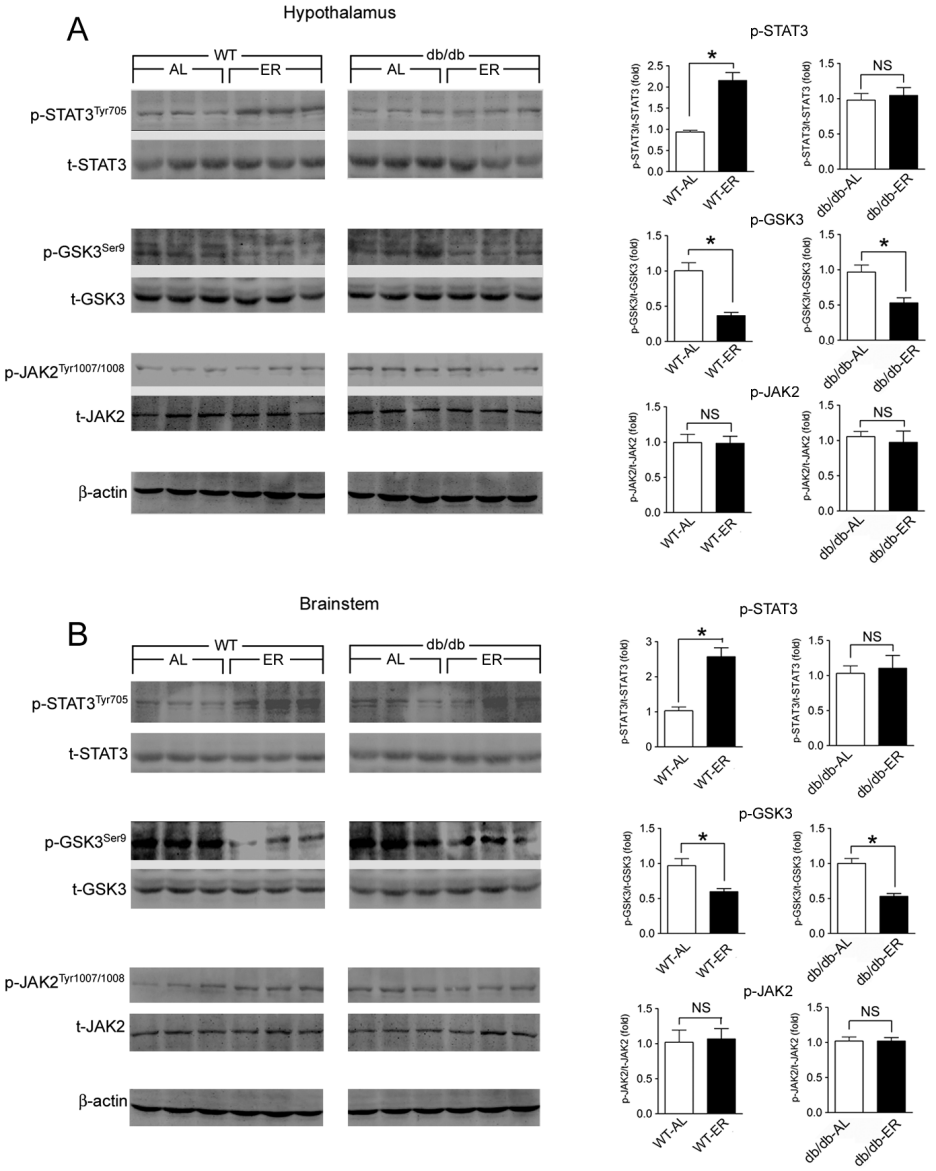

Figure 5. Effects of ER on leptin receptor–related signaling pathways in WT and *db/db* mice. Western blotting shows the phosphorylation of STAT3, GSK3 and JAK2 in mouse hypothalamus and brainstem, respectively. NS, no significance. n = 3 per group. **P* < 0.05 versus AL.

DISCUSSION

Emerging evidence suggests that the *FTO* gene is an obesity gene controlling food intake or energy expenditure, or both (1,2). In the present study, we demonstrated that ER was able to decrease both mRNA and protein expression of FTO in brain rather than in peripheral metabolic-sensitive tissues. Moreover, we found that the LepRb was essential for the effect of ER on FTO expression, and the STAT3 signaling pathway contributed to this process.

As far as we know, this is the first investigation addressing the FTO protein expression pattern in tissues and how FTO protein changes after ER. Consistent with several previous reports of FTO mRNA (10,12,35), we detected high mRNA expression of FTO in hypothalamus and brainstem. Additionally, we confirmed the high protein expression of FTO in hippocampus and brainstem. This finding indicates that the pattern of FTO protein expression is in accordance with the FTO mRNA expression pattern in brain. Furthermore, we found that the expression of the *FTO* gene in skeletal muscle and liver was higher than that in adipose tissue. Some researchers focused on adipose tissue to explore the relationship between the *FTO* gene and obesity. A previous report showed that FTO mRNA expression in subcutaneous adipose tissue is higher than in visceral adipose tissue (36), whereas another publication reported the reverse results (11). We showed that FTO mRNA expression in visceral adipose tissue was two- to three-fold higher than that in subcutaneous adipose tissue, whereas the FTO protein expressions were comparable in these two fat pads.

It should be noted that how the expression of FTO changes upon ER is still

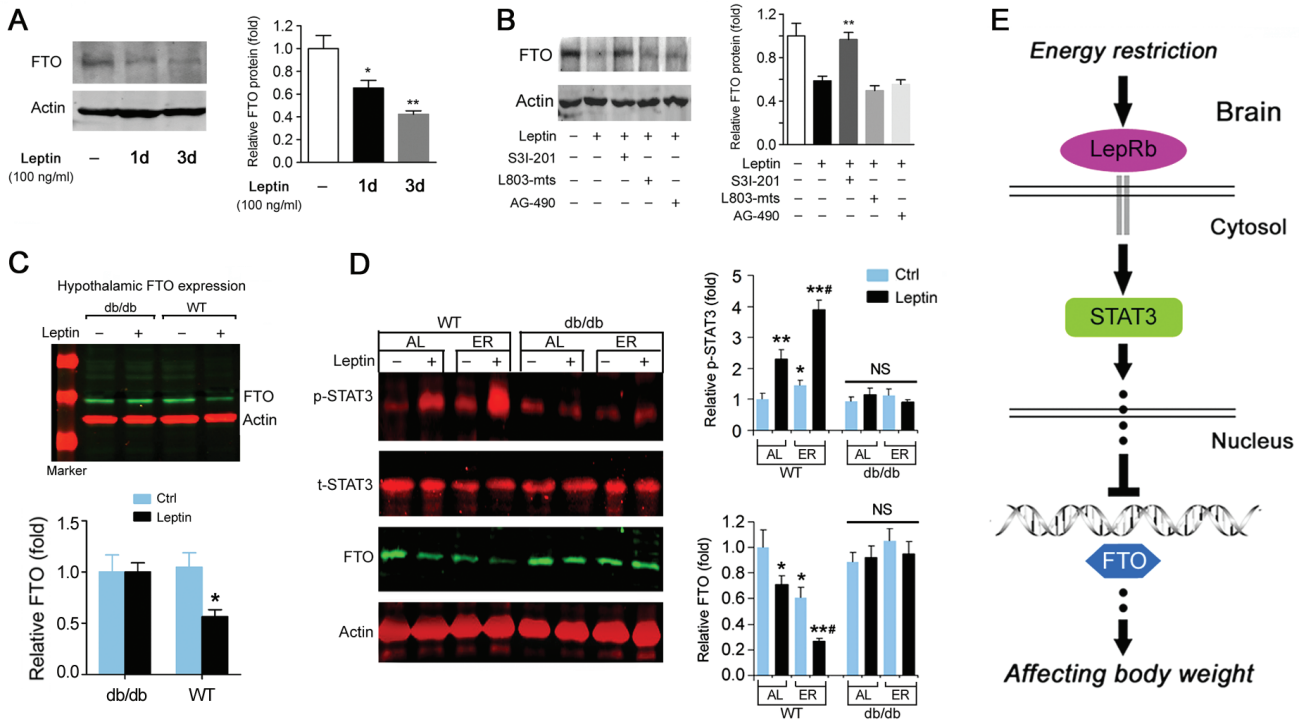

Figure 6. Leptin administration downregulates FTO *in vitro* and *in vivo*. (A) Leptin (100 ng/mL) decreases FTO expression in mouse ARH cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle control. 1d, 1 day; 3d, 3 days. (B) Leptin (100 ng/mL)-induced FTO downregulation in mouse ARH cultures is abolished by the STAT3-specific inhibitor S3I-201 (100 μmol/L), but not by the GSK-3–specific inhibitor L803-mts (20 μmol/L) or JAK2-specific inhibitor AG-490 (20 μmol/L). $^{**}P < 0.01$ versus leptin treated. (C) A single bolus leptin (1 μg) injection into lateral ventricle decreases hypothalamic FTO expression in WT but not *db/db* mice. $^{*}P < 0.01$ versus control (Crtl). (D) ER and leptin (1 μg) injection into the lateral ventricle activates the STAT3 pathway and downregulates FTO expression in WT but not *db/db* mice. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WT-AL. $^{\#}P < 0.01$ versus WT-ER. NS, no significance. (E) A proposed model of involvement of the leptin signaling pathway in brain FTO regulation.

disputable. Previous reports showed that FTO mRNA was downregulated in mouse hypothalamus (10,11) but upregulated in rat hypothalamus (12) after food deprivation (that is, fasting for 1–2 days). We considered it might be more reasonable that the rat and mouse share the same or similar regulating pattern of FTO expression. In this study, we found the decline of FTO mRNA and protein expression in hypothalamus and brainstem both in ER rats and mice. These results support the results of Gerken *et al.* (10) and Stratigopoulos *et al.* (11) in mice. However, the results may partly conflict with observations showing that FTO is upregulated during food deprivation in rats to some extent (12). However, the difference in treatment between our study and the above-mentioned studies should be emphasized. This study was designed to investigate the FTO expression change in calorically restricted animals for ≥8 weeks, whereas the previous studies focused on the influence of short-term food deprivation for 1–2 days on FTO expression. These differences may account for the different results.

Furthermore, we did not find any change of FTO mRNA or protein expressions in the peripheral metabolic-sensitive tissues in ER rats. These results suggest that the *FTO* gene in the peripheral metabolic tissues does not directly regulate metabolic processes under ER. Considering the high FTO expression in skeletal muscle and liver and that the *FTO* gene encodes a 2-oxoglutarate–dependent demethylase in the nuclei (10), even though we failed to find any change of *FTO* gene expression in peripheral metabolic tissues of ER rats, these results do not rule out that FTO may play a role in regulating biological function via posttranslational mechanisms in liver and muscle.

The most important finding of our study is the LepRb-dependent regulation of FTO expression during ER. It has been well established that hypothalamus and brainstem play key roles in generating homeostatic responses to regulate food intake and energy expenditure, and leptin is one of the most important hormones controlling the feeding and energy balance in these two brain areas (13,16,37). Moreover, several intracellular signaling pathways, such as STAT3, JAK2 and GSK3, were reported to contribute critically in the effects of leptin on neuropeptide production and body energy homeostasis (38–43). In our study, we found that the

FTO colocalizes with LepRb in hypothalamic and brainstem neurons. Thus, FTO might be regulated by leptin via LepRb-mediated intracellular signaling. To confirm this speculation, we tested whether ER can modulate FTO expression in LepRb-mutated *db/db* mice, a widely used obese animal model (16). In normal status, there is no difference of FTO expression between WT and *db/db* mice. FTO expression in hypothalamus and brainstem decreased significantly after 8 wks of ER in WT mice but not in *db/db* mice, indicating LepRb mutation impaired the effects of ER on FTO expression. We also found that the exogenous leptin treatment reduced FTO expression in *in vitro* ARH cultures. Moreover, stereotaxically intracerebroventricular microinjection of leptin indeed directly decreased the brain FTO expression in WT mice but not in *db/db* mice, indicating the brain FTO downregulation is LepRb dependent.

We noted that the circulating leptin level was lower (about 0.3–0.4-fold) in ER than in normal conditions (18,44). However, the LepRb expression in brain under ER conditions was increased significantly (18,19). The response to leptin under ER conditions is even more sensitive than under normal conditions. In fact, the ER rats or mice exhibited an increased response to leptin in brain (15,18,19,45). This enhanced leptin sensitivity under ER conditions was able to counteract the leptin resistance caused by aging and diet-induced obesity (15,18,19,45). Recently, Church *et al.* (46) demonstrated that circulating leptin levels were lower in FTO transgenic mice. Additionally, Stratigopoulos *et al.* (47) showed that FTO regulated LepRb trafficking to the cilium and subsequently modulated cellular response to leptin. Thus, the relationship between FTO and leptin/LepRb may be complex and needs to be further investigated.

Among the LepRb-related signal pathways, we demonstrated that only the STAT3 activation induced by ER was abolished in *db/db* mice (see Figure 5), whereas the effects of ER on JAK2 and GSK3 pathways did not change in *db/db* mice. Leptin treatment directly downregulated FTO in ARH cultures *in vitro*, which could be abolished by the STAT3-specific inhibitor but not the GSK-3 or JAK2 inhibitor (Figure 6B). Moreover, leptin injection reduced FTO expression (Figure 6C) and activated the STAT3 signaling pathway (Figure 6D) in WT but not in *db/db* mice *in vivo*. Therefore, the change trends of FTO and p-STAT3 make us believe that the LepRb-STAT3 signaling pathway is critically involved in the downregulation of brain FTO during ER. Tung *et al.* (48) demonstrated that overexpression of FTO in rat ARH using adeno-associated virus not only decreased food intake, but also resulted in a four-fold increase in the mRNA levels of STAT3. They also considered that the STAT3 might be a possible candidate for the mediation of FTO actions.

In conclusion, we have demonstrated that ER can decrease FTO mRNA and protein expression in both rat and mouse brains. The LepRb-STAT3 signaling pathway is involved in this process, not only evidenced by the absence of FTO downregulation and impairment of STAT3 activation in food-restricted *db/db* mice, but also evidenced by exogenous leptin activating the STAT3 signaling pathway and decreasing FTO expression *in vitro* and *in vivo*. Therefore, these findings suggest an involvement of the LepRb-STAT3 signaling pathway in brain FTO downregulation by ER.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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