O-GlcNAc Transferase Enables AgRP Neurons to Suppress Browning of White Fat

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SUMMARY

Induction of beige cells causes the browning of white fat and improves energy metabolism. However, the central mechanism that controls adipose tissue browning and its physiological relevance are largely unknown. Here, we demonstrate that fasting and chemical-genetic activation of orexigenic AgRP neurons in the hypothalamus suppress the browning of white fat. O-linked β-N-acetylglucosamine (O-GlcNAc) modification of cytoplasmic and nuclear proteins regulates fundamental cellular processes. The levels of O-GlcNAc transferase (OGT) and O-GlcNAc modification are enriched in AgRP neurons and are elevated by fasting. Genetic ablation of OGT in AgRP neurons inhibits neuronal excitability through the voltage-dependent potassium channel, promotes white adipose tissue browning, and protects mice against diet-induced obesity and insulin resistance. These data reveal adipose tissue browning as a highly dynamic physiological process under central control, in which O-GlcNAc signaling in AgRP neurons is essential for suppressing thermogenesis to conserve energy in response to fasting.

INTRODUCTION

Overweight and obesity develop when energy intake exceeds energy expenditure, storing excess calories in the adipose tissue (Spiegelman and Flier, 2001). The adipose organ comprises white (WAT) and brown adipose tissues (BAT). WAT primarily stores energy as triglycerides and its excess and dysfunction lie at the core of obesity and associated metabolic disorders. In contrast, BAT-mediated adaptive thermogenesis dissipates chemical energy as heat, and protects against obesity in both rodents and humans (Cinti, 2012; Kajimura and Saito, 2013; Nedergaard et al., 2010; Smorlesi et al., 2012). Emerging evidence has demonstrated that “brown-like” adipocytes, so-called beige/brite cells, exist in specific WAT depots and differ from classic brown adipocytes in their origin and molecular identity (Petrovic et al., 2010; Rosen and Spiegelman, 2014; Wu et al., 2012). Multiple intrinsic factors and secreted molecules have been identified that modulate the development and function of beige/brite adipocytes and thus metabolic health in animals (Bartelt and Heeren, 2013; Wu et al., 2013). However, whether and how the central nervous system controls WAT browning is almost completely unknown.

Orexigenic neurons expressing agouti-related protein (AgRP)/neuropeptide Y (NPY) and anorexigenic neurons expressing proopiomelanocortin (POMC) reside in the arcuate nucleus of the hypothalamus. These neurons are regulated by peripheral hormones and nutrients and are critical for maintenance of energy homeostasis and glucose metabolism. During food deprivation, AgRP neurons are strongly activated to promote hunger (Hahn et al., 1998; Liu et al., 2012; Takahashi and Cone, 2005), an effect vastly mediated by ghrelin signaling in these neurons (Andrews et al., 2008; Chen et al., 2004; Yang et al., 2011). Despite the involvement of other hypothalamic areas in the control of thermogenesis in classic BAT (Nogueiras et al., 2008; Scherer and Buettner, 2011; Yasuda et al., 2004), it is not known whether hunger-promoting AgRP neurons are involved in the control of adaptive thermogenesis and/or browning of WAT.

Thousands of cytoplasmic and nuclear proteins are modified by a single O-linked β-N-acetylglucosamine (O-GlcNAc) moiety at serine or threonine residues, termed O-GlcNAcylation (Hart et al., 2007; Torres and Hart, 1984). This dynamic and reversible modification is emerging as a key regulator of diverse cellular processes, such as signal transduction, transcription, translation, and proteasomal degradation (Love and Hanover, 2005; Ruan et al., 2013a; Yang et al., 2002). Perturbations in protein O-GlcNAcylation are implicated in various human diseases including diabetes mellitus, neurodegeneration, and cancer.
Here, we show that OGT expression is enriched in hypothalamic AgRP neurons and induced by fasting and ghrelin. Pharmacogenetical activation of AgRP neurons suppresses the thermogenic program in WAT, while the selective knockout of Ogт in AgRP neurons inhibits neuronal activity, promotes WAT browning, and protects mice against diet-induced obesity.

RESULTS

Fasting Suppresses Thermogenic Program in WAT

A major component of energy homeostasis is to adjust energy expenditure according to the level of energy intake (Apfelbaum et al., 1971; Shibata and Bukowiecki, 1987; Welle and Campbell, 1983). Given that WAT browning is an emerging regulator of energy expenditure, we test whether food availability regulates the browning process. Four adipose depots including classic BAT, gonadal WAT (gWAT), and two major depots that have the potential of browning—retroperitoneal and inguinal WAT (rWAT)—from ad libitum fed and 24 hr-fasted mice were collected (Fisher et al., 2012; Guerra et al., 1998; Nedergaard and Cannon, 2013). Fasting reduced total RNA level in most depots (Figure S1A available online), thus thermogenic gene expression was determined by relative real-time PCR and calculated as total level per depot (Nedergaard and Cannon, 2013). Ucp1 expression was significant decreased in BAT and rWAT (Figures 1A and 1B) and showed a tendency of reduction in iWAT and gWAT in fasted mice as compared to fed controls.

Figure 1. Fasting and AgRP Neurons Suppress WAT Browning

(A–C) Expression of thermogenic genes in different fat depots from ad libitum fed or 24 hr-fasted mice (n = 5). Same amount of RNA was used for reverse transcription followed by real-time PCR. Gene expression was first normalized to 36B4 and then relative mRNA amount per depot was calculated based on total RNA levels (Figure S1A). Total levels of thermogenic genes per depot in BAT (A), rWAT (B), and iWAT (C) are shown. (D–H) Mice were either fed ad libitum or fasted overnight at room temperature (RT) or 4°C. (D) Expression of Ucp1 transcript in different fat depots (n = 5). Values represent fold change relative to the RT/Fed group within each fat depot. (E) Immunoblotting showing protein levels of Ucp1, TH, and Uchl1. Densitometric analyses are shown at the bottom. (F–H) NE levels in (F) BAT, (G) iWAT, and (H) rWAT. Data are shown as mean ± SEM. *p < 0.01; **p < 0.01 by unpaired Student’s t test.

See also Figure S1.
The expression of other thermogenic genes including Ppargc1a, Pdirm16, Cidea, and Dio2 during fasting was significantly suppressed in rWAT, but mainly unchanged in BAT, iWAT, and gWAT (Figures 1A–1C and S1B). These data suggest that rWAT is the major depot responsible for the inhibitory effect of fasting on thermogenesis.

Cold exposure has been extensively shown as a physiological stimulator of BAT activation and WAT browning (Cinti, 2012). However, cold exposure did not efficiently induce Ucp1 transcription in any fat depots when animals were deprived of food, indicating that fasting diminishes the effect of cold on thermogenesis (Figures 1D and S1C). When we examined the levels of Ucp1 protein in these fat depots, we found that fasting downregulated Ucp1 in rWAT but not in BAT and iWAT (Figure 1E). Sympathetic outflow to BAT and WAT controls the expression of thermogenic genes and heat production in brown and beige fat (Harms and Seale, 2013). Thus we hypothesized that changes in sympathetic nerve activity in response to stimuli determine the thermogenic program in different depots. Fasting increased levels of norepinephrine (NE) in serum (Figure S1D), BAT, and iWAT (Figures 1F and 1G). However, NE level only in rWAT was decreased during fasting (Figure 1H), correlated with the reduction in thermogenic gene expression. Fasting also dampened the induction of NE level in rWAT by cold (Figure 1H). In addition, fasting downregulated while cold upregulated the levels of tyrosine hydroxylase (TH), a marker of sympathetic nerve, and ubiquitin carboxyl-terminal esterase L1 (Uch1), a general marker for peripheral neurons in rWAT (Figure 1E) (Burgi et al., 2011; Wilkinson et al., 1989). Taken together, these data suggest that fasting controls sympathetic outflow and regulates browning in rWAT.

Acute Activation of AgRP Neurons Suppresses Thermogenic Program in WAT

Orexigenic AgRP/NPY neurons in the hypothalamus are critical for energy homeostasis and glucose metabolism in response to nutrient and hormonal cues (Belgardt et al., 2009). During fasting, the activation of AgRP neurons provokes animals to seek food and simultaneously suppresses energy expenditure (Dietrich and Horvath, 2012; Small et al., 2001). To determine whether AgRP neurons regulate WAT browning, we took advantage of a chemical genetics approach that allows for acute, cell type-specific control of neuronal activity in vivo. Specifically, we generated mice expressing the cation channel Trpv1 only in AgRP neurons (Figure 2A) (Arenkiel et al., 2008). Capsaicin is a Trpv1 agonist that will induce the depolarization and activation of AgRP neurons in Trpv1−/−;AgRP-Cre−;R26Trpv1 mice. Acute activation of AgRP neurons by systemic injection of capsaicin (10 mg/kg, intraperitoneally [i.p.]) for only 1 hr strongly inhibited the expression of thermogenic genes including Ucp1, Ppargc1a, Pdirm16, and Cidea in rWAT, and to a less extent in iWAT, but not in classic BAT or visceral gWAT in Trpv1−/−;AgRP-Cre−;R26Trpv1 mice (Figure 2B). Activation of AgRP neurons significantly reduced energy expenditure in Trpv1−/−;AgRP-Cre−;R26Trpv1 mice, compared to Trpv1−/−;AgRP-Cre−;R26Trpv1 mice (Figure 2C). Food was removed during these studies to eliminate the effect of diet-induced thermogenesis. Thus far, these data indicate that acute activation of AgRP neurons decreases energy expenditure and thermogenic gene expression profile in selected fat depots.

To test the physiological importance of AgRP neuron-regulated browning, capsaicin-injected mice were immediately placed at 4°C. Core body temperature dropped lower in Trpv1−/−;AgRP-Cre−;R26Trpv1 mice (Figure 2D), suggesting that AgRP activation inhibits cold-induced thermogenesis. Gene expression analyses showed that cold promoted Ucp1 transcription in BAT (Figure 2E), but not in rWAT (Figure 2F) of Trpv1−/−;AgRP-Cre−;R26Trpv1 mice, indicating that thermogenic program in rWAT is specifically inhibited by AgRP neurons. Acute activation of AgRP neurons did not change serum levels of NE (Figure 2G). However, we observed a reduction of NE level specifically in rWAT of Trpv1−/−;AgRP-Cre−;R26Trpv1 mice (Figure 2H). Treatment with a selective β3 agonist, CL-316, 243 rescued the decrease in body temperature and Ucp1 expression in rWAT of capsaicin-injected Trpv1−/−;AgRP-Cre−;R26Trpv1 mice (Figures 2I and 2J). These data demonstrate that acute activation of hunger-promoting AgRP neurons suppresses browning in rWAT by regulating sympathetic activity.

OGT Controls AgRP Neuronal Activity

We then sought to determine possible nutrient sensors in AgRP neurons that respond to fasting. Previously, we have shown that O-GlcNAcylation of insulin signaling proteins and transcriptional regulators in peripheral tissues is important for glucose and lipid metabolism (Li et al., 2013; Ruan et al., 2012, 2013b; Yang et al., 2008). Expression of OGT and overall O-GlcNAc levels (Figures S2A and S2B) in the hypothalamus are significantly higher than peripheral tissues such as liver, WAT, and muscle. However, little is known about the role of O-GlcNAc modification in the hypothalamic regulation of metabolism. To determine the relative levels of Ogt transcripts in AgRP neurons, we isolated AgRP neuron-specific ribosome-associated mRNAs from the arcuate nucleus of AgRP-Cre−;R262×HA mice by immunoprecipitation of the actively translating polyribosomes that were tagged with hemagglutinin (HA) epitope (Sanz et al., 2009). Ogt transcripts showed a 4-fold enrichment in AgRP neurons (Figure 3A). Consistently, immunohistochemistry demonstrated that a subset of AgRP/NPY neurons have relatively high levels of OGT proteins and O-GlcNAcylation (Figures 3B and 3C). Food deprivation for 24 hr increased OGT expression and O-GlcNAc levels in AgRP neurons (Figures 3B and 3C). Ghrelin is a hormone released by the empty stomach that promotes hunger by activating AgRP neurons in the hypothalamus (Andrews et al., 2008; Chen et al., 2004; Liu et al., 2012; Luquet et al., 2007; Wiedmer et al., 2011). We found that ghrelin increased O-GlcNAc levels in AgRP neurons (Figure 3D) and reduced levels of Ucp1 protein in rWAT (Figure S2C). These data point to the possibility that O-GlcNAcylation functions as a fasting signal in AgRP neurons.

To determine the physiological role of O-GlcNAc signaling in AgRP neurons, we generated AgRP neuron-specific Ogt knockout (KO) mice (Figure S2D). Immunofluorescent staining of Ogt on Npy-hrGFP hypothalamus showed that OGT was specifically deleted in AgRP neurons (Figure S2E). Real-time PCR and western blot analyses confirmed that OGT was not ectopically deleted in the whole hypothalamus, cortex, or other peripheral metabolic tissues (Figures S2F and S2G). To identify AgRP neurons during electrophysiological studies, AgRP-Ogt KO mice were cross-bred into the Npy-hrGFP background (Figure 3E).
Figure 2. Acute Activation of AgRP Neurons Suppresses the Thermogenic Program in WAT

(A) Ten mg/kg body weight of capsaicin was injected to Trpv1−/−;AgRP-Cre−;R26Exon12+/− control (CT) and KO mice (Figure 3F). However, the spontaneous firing rate was reduced in KO mice (Figures 3G and 3H). Whole-cell current clamp measurements demonstrated that the membrane potential of AgRP neurons was similar between control (CT) and KO mice (Figure 3F). However, the spontaneous firing rate was reduced in KO mice (Figures 3G and 3H).

OGT Modulates the Potassium Channel in AgRP Neurons

Voltage-dependent potassium (Kv) channels mediate the repolarization and after-hyperpolarization phases of action potential, and defects in Kv current lead to dampened activity but intact membrane potential in neurons (Bean, 2007). Electrophysiological analyses showed a reduction in outward K+ current in AgRP neurons from KO mice compared to CT mice (Figures 4A and 4B). The K+ current in AgRP neurons was slowly inactivated, suggesting a possible involvement of delayed rectifier Kv channels. Kcnq3 (Kv7.3), mediating delayed rectifier Kv current, is expressed in AgRP neurons (Ren et al., 2012). We found that OGT interacted with Kcnq3 in the hypothalamus, and fasting enhanced their interaction (Figure 4C). Kcnq3 has been shown to be O-GlcNAc-modified at murine synapse (Trinidad et al., 2012), and mutation of the O-GlcNAc site Threonine 655 to Alanine (T655A) almost abolished O-GlcNAcylation on Kcnq3 (Figure 4D). Then we assessed Kcnq3 activity and found that T655A mutant channel showed a significant reduction in K+ current compared to the wild-type Kcnq3 (Figures 4E and 4F), suggesting that O-GlcNAcylation of this Kv channel is a key factor in dampening the activity of AgRP neurons.
regulatory mechanism underlying cellular excitability. Taken together, AgRP-Ogt KO mice are intrinsically defective in the activity of Kv channels and neuronal firing, serving as a model to study metabolic roles of AgRP neurons.

**Ogt Knockout in AgRP Neurons Promotes Thermogenic Program in WAT**

Acute activation of AgRP neurons specifically suppressed rWAT browning (Figure 2), thus we hypothesized that impaired AgRP neuronal activity in AgRP-Ogt knockout mice would induce a thermogenic gene program in rWAT. As expected, the levels of Ucp1 and Cidea mRNAs were significantly increased in rWAT (Figure 5A), but remained unchanged in BAT of KO mice as compared to CT mice (Figure 5B). Consistently, uncoupled oxygen consumption rate (OCR) in BAT remained the same, while uncoupled OCR in rWAT was higher, when comparing KO mice to CT mice (Figure 5C). Blocking the β3 adrenergic receptor by SR59230A restored Ucp1 expression in rWAT (Figure 5D), strengthening the notion that AgRP neurons control rWAT browning through SNS.

BAT of KO mice remained susceptible to the suppression of thermogenic genes by fasting (Figure 5E, compared to Figure 1A). In contrast, this suppression was abolished in rWAT of KO mice (Figure 5F, compared to Figure 1B). Ghrelin also failed to downregulate Ucp1 protein levels in rWAT of KO mice (Figure 5G). These data demonstrate that O-GlcNAc signaling in AgRP neurons is required for the regulation of WAT browning by fasting and ghrelin. As a result, AgRP-Ogt KO mice showed less reduction in energy expenditure than CT mice during the first 24 hr of fasting (Figure 5G) with concomitant increase in weight loss (Figure 5H). Nevertheless, there was no significant reduction in body weight of ad libitum KO mice (Figure S3B).

The defect in AgRP neuronal activity is normally associated with decreased feeding behavior (Dietrich et al., 2010). However, there was no change in food intake in AgRP-Ogt KO mice (Figure S3C). This is possibly due to the overproduction of Agrp and Npy transcripts via unknown compensatory mechanisms (Figure S3D), and/or the OGT-positive subset of AgRP neurons does not directly modulate feeding. Cold exposure did not affect OGT expression in AgRP neurons (Figure S3E), and AgRP-Ogt KO mice and control mice under fed conditions showed similar core body temperature during cold challenge (Figure S3F), indicating that the silencing of AgRP neurons either by feeding or by OGT deletion abolishes the impact of this neural circuit on cold-induced thermogenesis.

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**Figure 3. OGT Is Required for AgRP Neuronal Activity**
(A) Ribosome-associated mRNAs were isolated from the arcuate nucleus of AgRP-Cre+; RPL22HA mice, and real-time PCR was performed to determine the enrichment of Ogt transcripts in HA immunoprecipitation compared to the input (n = 4). Agrp and Pomc transcripts were used as controls.
(B) Immunostaining of OGT in the hypothalamus of fed and overnight-fasted Npy-hrGFP mice.
(C) Immunostaining of O-GlcNAc in the hypothalamus of fed and overnight-fasted Npy-hrGFP mice.
(D) Immunostaining of O-GlcNAc in the hypothalamus of Npy-hrGFP mice injected with saline or 120 mmol/kg body weight of ghrelin for 1 hr.
(E) AgRP-Cre;Ogtflx (CT) and AgRP-Cre;Ogtflx (KO) mice were crossbred onto Npy-hrGFP background for the whole-cell current-clamp recordings.
(F) Basal membrane potential of AgRP neurons (n = 19).
(G) Representative tracing of action potentials of AgRP neurons.
(H) Firing rate of AgRP neurons (n = 19). Data are shown as mean ± SEM. *p < 0.05 by unpaired Student’s t test. 3V, 3rd ventricle. Scale bar represents 50 μm. See also Figure S2.
Data are shown as mean ± SEM. *p < 0.05 by unpaired Student’s t test.

**DISCUSSION**

Studies in the past few years have greatly expanded our knowledge of beige adipocytes. There is no doubt that beige fat is metabolically important, especially during cold exposure and nutrient overload. Stimulating the browning process protects mice from diet-induced obesity, whereas the ablation of beige adipocytes causes metabolic dysfunction (Cohen et al., 2014; Seale et al., 2011). In this study, we identify fasting as a negative, physiological regulator of the thermogenic program in beige adipocytes. Intriguingly, fasting diminishes the effect of cold exposure on thermogenesis, suggesting that fasting is a predominant regulator of browning in order to conserve energy for survival.

The hypothalamus has been long proposed to regulate adaptive thermogenesis in BAT, independently on its regulation on food intake (Kong et al., 2012; Vogt and Brüning, 2013). Neuropeptides AgRP and NPY have been shown to inhibit BAT function, while α-MSH increases SNS activity and BAT function (Shi et al., 2013; Yasuda et al., 2004). It is not known whether these circuits also control WAT browning. In this study, we demonstrate that chemical-genetic activation of AgRP neurons acutely suppresses the thermogenic program in beige adipocytes, demonstrating that white fat browning is a highly dynamic and reversible process. Conversely, impairment in AgRP neuronal activity abolishes fasting-mediated inhibition of WAT browning. These results reveal the dynamic nature of WAT browning and identify a specific neuronal population that negatively regulates this process.

**Ogt Knockout in AgRP Neurons Protects against Diet-Induced Obesity and Insulin Resistance**

Next, we challenged the mice with high fat diet (HFD). Consistent with the findings in mice on NC, thermogenic genes including Ucp1, Pparc1a, Pdm16, and Cidea were dramatically increased in rWAT of AgRP-Ogt KO mice (Figure 6A). There were more “brown-like” adipocytes and less fat exist in rWAT of KO mice (Figures 6B and 6C). Although BAT and iWAT showed a reduction in fat weight and content (Figures 6B and 6C), the expression of thermogenic genes was comparable between KO and CT groups (Figure 6A). We did not observe any changes in weight, morphology, or gene expression in gWAT (Figures 6A–6C). We also observed that protein levels of Ucp1 and tyrosine hydroxylase were markedly elevated in rWAT of KO mice (Figure 6D). These mice also exhibited increased NE levels in rWAT but not in BAT or iWAT (Figure 6E). These data demonstrate that Ogt knockout in AgRP neurons selectively activates browning in rWAT of mice fed on HFD.

Consistent with the activated thermogenic program, heat production in KO mice was significantly increased compared to CT mice, as demonstrated by the metabolic cage study (Figure 6F). As a result, both female and male KO mice gained significantly less body weight and fat mass compared to CT mice (Figures 7A, 7B, S4A, and S4B), despite consuming a similar amount of HFD (Figure 7C). Although levels of fasting glucose and insulin were not significantly different (Figures 7D, 7E, S4C, and S4D), there was a reduction in the values of the homeostasis model assessment of insulin resistance (HOMA-IR) in both female and male KO mice (Figures 7F and S4E). Consistent with these observations, glucose tolerance test and insulin tolerance test showed that AgRP-Ogt KO mice were more glucose-tolerant and insulin-sensitive than CT mice (Figures 7G, 7H, and S4F). Taken together, these data reveal that Ogt deficiency in AgRP neurons increases WAT browning and protects mice from diet-induced obesity and insulin resistance.
Fasting and AgRP neuronal activation appear to regulate browning preferentially in rWAT, to a much less extent in other WAT depots but not in BAT. We demonstrate that uniquely in rWAT, sympathetic nerve activity correlates with thermogenic gene expression and browning during cold and fasting stimuli. In this regard, mapping the neuronal circuits linking AgRP neurons to sympathetic innervation onto different WAT depots would provide further insights. It is also possible that other mechanisms may mediate the effect of AgRP neurons on WAT browning. Although as a relative small depot, rWAT responds much quicker than BAT and other WAT depots after cold exposure or food deprivation. This suggests that browning in rWAT may function as a first line of defense to maintain energy homeostasis when food availability and environmental temperature are fluctuant.

O-GlcNAc signaling has long been proposed as a nutrient sensor in multiple peripheral tissues. Hyperglycemia-associated elevation in O-GlcNAc levels mediates many aspects of gluco-toxicity (Ruan et al., 2013b). On the other hand, O-GlcNAc levels can also be increased by glucose deprivation in several cell types (Cheung and Hart, 2008; Kang et al., 2009; Taylor et al., 2008). Consistent with the latter observations, we demonstrate that OGT expression and O-GlcNAc modification increase in AgRP neurons in response to fasting and ghrelin, although the molecular mechanism has yet to be defined. Genetic ablation

**Figure 5. Loss of Ogt in AgRP Neurons Promotes Browning and Improves Glucose Metabolism in Mice Fed on Normal Chow**

(A and B) Expression of thermogenic genes in rWAT (A) and BAT (B) of 6-month-old female mice (n = 4).

(C) Oxygen consumption rate of BAT and rWAT in the presence of oligomycin, an ATPase inhibitor (n = 8).

(D) Expression of Ucp1 in rWAT from mice treated with 3 days of saline or SR59230A (n = 4–6).

(E and F) Expression of thermogenic genes in BAT (E) and rWAT (F) from fed and 24 hr-fasted AgRP-Ogt KO female mice (n = 3–4). Total amounts of mRNA were calculated based on relative mRNA levels and total amounts of RNA isolated.

(G and H) Loss of energy expenditure (G) and body weight (H) in CT and KO female mice after fasting for 24 hr (n = 6–15).

(I) Expression of gluconeogenic genes in liver of 6-month-old female mice (n = 3–4).

(J) Pyruvate tolerance test in 5-month-old female mice (n = 4–7). Insert, area under curve (AUC).

(K) Glucose tolerance test in 5-month-old female mice (n = 8–12). Insert, AUC. Data are shown as mean ± SEM. *p < 0.05 by unpaired Student’s t test. See also Figure S3.
of OGT in AgRP neurons promotes WAT browning, thus leading to improved glucose and energy metabolism. Mouse models with defective BAT often fail to maintain their body temperature upon cold exposure (Feldmann et al., 2009). However, OGT expression in AgRP is not affected by cold, and AgRP-Ogt KO mice maintain a normal core temperature upon cold exposure. These observations prompt the hypothesis that brown fat primarily maintains homeothermy to combat cold, whereas beige fat regulates energy metabolism in response to nutrient stress. This notion is supported by the recent finding that PRDM16 ablation in beige fat does not affect body temperature in mice (Cohen et al., 2014).

Neuronal circuits in the arcuate nucleus of the hypothalamus are relatively tolerant to perturbations, especially during developmental and neonatal stages. Neonatal ablation of AgRP neurons has minimal effects on feeding, although ablation of these neurons in adults causes rapid starvation (Luquet et al., 2005). Genetic knockout of Agrp gene in mice also does not affect food intake (Qian et al., 2002). These data suggest that neuronal plasticity can compensate for the loss of Agrp gene or AgRP neurons. In our study, the deletion of Ogt in AgRP neurons occurs early during development. Although neuronal activity is decreased in AgRP-Ogt KO mice, the expression of Agrp and Npy transcripts is elevated, which may contribute to the maintenance of normal food intake.

Hunger and cold are two life-history variables during the development and evolution of mammals. We have observed that food deprivation (the negative regulator) dominates over cold exposure (the positive regulator) in the central control of WAT browning. This regulatory system may be evolutionarily important as it can reduce heat production to maintain energy balance during fasting. Modulating the hypothalamic control of WAT browning
represents a potential strategy to combat obesity and associated morbidity.

**EXPERIMENTAL PROCEDURES**

**Mice**

Ogt-floxed mice on C57BL/6 background (Shafi et al., 2000) were kindly provided by Dr. Steven Jones (University of Louisville). AgRP-Cre mice, kindly donated by Alison Xu (University of California San Francisco), have been maintained in our colony on a mixed background (Xu et al., 2005). Trpv1tm1Jul/J mice (#003770), Gt(ROSA)26Sortm1(Trpv1,ECFP)Mde314, and Npy-hrGFP mice (#006417), which express humanized Renilla GFP under the control of the mouse Npy promoter, were from Jackson Laboratory. To express Trpv1 selectively in AgRP neurons, we have bred both Trpv1 colonies to a second AgRP- mouse. All animals were housed for at least 1 week for environmental habituation, and food consumption was weighed every morning for 7 consecutive days. For the metabolic cage study, mice were acclimated in metabolic chambers (TSE Systems) for 3 days and then gas exchange, food intake, and ambulatory activity were recorded for another 3 days. Heat production was calculated and tissues were collected 2 hr after the final injection. All mice were anesthetized with isoflurane and sacrificed by decapitation. The brain was gently removed from the skull and chilled in 4°C for subsequent experiments.

**Metabolic Assays**

Body weights were recorded every week. Body composition was assessed using an EchoMRI system. For food intake measurement, mice were individually housed for at least 1 week for environmental habituation, and food consumption was weighed every morning for 7 consecutive days. For the metabolic cage study, mice were acclimated in metabolic chambers (TSE Systems) for 3 days and then gas exchange, food intake, and ambulatory activity were recorded continuously for another 3 days. Heat production was calculated and adjusted to body weight (Tscho¨p et al., 2012). Body temperature was measured rectally using a thermo-coupler (Physitemp). For pyruvate-, glucose-, and insulin-tolerance tests, 16-hour-fasted mice were injected with intraperitoneally with sodium pyruvate (1.5 g/kg body weight) or glucose (1.5 g/kg body weight); 6 hr fasted mice were injected with insulin (1 U/kg body weight). Blood glucose from tail-vein blood collected at the designated times was measured using a Nova Max Glucometer. Insulin (Millipore) and norepinephrine (Abnova) were determined using ELISA kits.

**Electrophysiology**

Mice were anesthetized with isoflurane and sacrificed by decapitation. The brain was gently removed from the skull and chilled in 4°C for subsequent experiments. For the metabolic cage study, mice were acclimated in metabolic chambers (TSE Systems) for 3 days and then gas exchange, food intake, and ambulatory activity were recorded continuously for another 3 days. Heat production was calculated and adjusted to body weight (Tscho¨p et al., 2012). Body temperature was measured rectally using a thermo-coupler (Physitemp). For pyruvate-, glucose-, and insulin-tolerance tests, 16-hour-fasted mice were injected with intraperitoneally with sodium pyruvate (1.5 g/kg body weight) or glucose (1.5 g/kg body weight); 6 hr fasted mice were injected with insulin (1 U/kg body weight). Blood glucose from tail-vein blood collected at the designated times was measured using a Nova Max Glucometer. Insulin (Millipore) and norepinephrine (Abnova) were determined using ELISA kits.
Ribosome RNA Enrichment

For ribosome profiling, the methods used here were in accordance with the original description of the animal model with minor modifications (Dietrich et al., 2013; Sanz et al., 2009). Fifty-day-old mice (from both genders) were sacrificed and five or six hypothalami were pooled for each n. A total of n = 4 was used. After RNA isolation, we obtained ~25 ng of RNA per sample. Only samples with high enrichment for Agrp and Npy were used for Ogt gene expression analyzes.

RNA and Real-Time PCR

Total RNA was extracted from mouse tissues using TRIzol reagent (Invitrogen). cDNA was reverse transcribed (Bio-Rad) and amplified with SYBR Green Supermix (Bio-Rad) using a LightCycler 480 real-time PCR system (Roche). All data were normalized to the expression of 18 s and 36b4. Primer sequences are available on request. When comparing gene expression between fed and fasted animals, total amounts of mRNA were calculated based on relative mRNA levels and total amounts of RNA isolated from specific depots (Nedergaard and Cannon, 2013).

Cell Culture

HEK293T cells were cultured in DMEM with 10% fetal bovine serum (FBS). Transfection was performed using FuGENE HD (Promega) according to the manufacturer’s instructions. For immunoprecipitation, whole-cell lysates were mixed with the Myc or Kcnq3 antibody and precipitated by Protein A/G agarose beads (Santa Cruz).

Western Blotting

Anti-OGT (ab96718), anti-O-GlcNac (RL2, ab2739), and anti-UCP1 (ab10983) were from Abcam. Anti-tyrosine hydroxylase (#2792) and anti-UCHL1 (#3524) were from Cell Signaling Technology. Anti-Myc (ac-40) was from Santa Cruz Biotechnology. Anti-Kcnq3 (NB9174102) was from Novus. Tissues were lysed in buffer containing 1% Nonidet P-40, 50 mM Tris × HCl, 0.1 mM EDTA, 150 mM NaCl, proteinase inhibitors and protein phosphatase inhibitors. Equal amounts of protein lysate were electrophoresed on SDS-PAGE gels and transferred to PVDF membrane. Primary antibodies were incubated at 4°C for overnight. Western blotting was visualized by peroxidase conjugated secondary antibodies and ECL chemiluminescent substrate.

Histology

Mice were anesthetized for intracardiac perfusion of PBS, followed by 4% paraformaldehyde. Brain and adipose depots were dissected and postfixed in 4% paraformaldehyde overnight. Coronal brain sections (50 μm) were prepared using a vibrating microtome. Paraffin sections of fat tissues were stained with hematoxylin and eosin staining (H&E). For immunofluorescence, tissue paraffin sections were from Abcam. Anti-tyrosine hydroxylase (#2792) and anti-UCHL1 (#3524) were from Cell Signaling Technology. Anti-Myc (ac-40) was from Santa Cruz Biotechnology. Anti-Kcnq3 (NB9174102) was from Novus. Tissues were lysed in buffer containing 1% Nonidet P-40, 50 mM Tris × HCl, 0.1 mM EDTA, 150 mM NaCl, proteinase inhibitors and protein phosphatase inhibitors. Equal amounts of protein lysate were electrophoresed on SDS-PAGE gels and transferred to PVDF membrane. Primary antibodies were incubated at 4°C for overnight. Western blotting was visualized by peroxidase conjugated secondary antibodies and ECL chemiluminescent substrate.

Statistical Analyses

Results are shown as mean ± SEM. The comparisons were carried out using two-tailed unpaired Student’s t test or one-way ANOVA followed by post hoc comparisons using Tukey corrections.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.09.010.

AUTHOR CONTRIBUTIONS

H.-B.R. designed and performed most of the experiments. Z.-W.L. designed and performed electrophysiological studies. M.O.D. and T.L.H. generated essential animal models and designed and executed experiments. M.R.Z. performed ribosome-mRNA profiling. M.L., J.P.S., K.Z., R.Y., and J.W. assisted in experiments. X.Y. conceived, designed, and supervised the project. H.-B.R. and X.Y. wrote the manuscript.

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REFERENCES


Figure S1. WAT Browning during Fasting, Related to Figure 1

(A and B) Mice (n = 5) were fed ad libitum or fasted for 24h. (A) Total RNA levels per depot. (B) Total levels of thermogenic genes in gWAT.

(C and D) Mice (n = 5) were either fed ad libitum or fasted for 24h at room temperature (RT) or 4°C. (C) Total RNA levels per depot. (D) Serum levels of NE. Data are shown as mean ± SEM. *p < 0.05; **p < 0.01; and ***p < 0.001 by unpaired Student’s t test.
Figure S2. Generation of AgRP-Ogt Knockout Mice, Related to Figure 3
(A) Levels of Ogt transcript in different tissues (n = 8).
(B) OGT proteins and O-GlcNAc levels in different tissues shown by immunoblotting.
(C) Expression of Ucp1 protein in rWAT of wild-type mice treated with saline or ghrelin for 1h.
(D) Breeding strategy to produce control (CT, AgRP-Cre;Ogt-flox) and knockout (KO, AgRP-Cre+;Ogt-flox) mice.
(E) Immunostaining of OGT in the hypothalamus of Npy-hrGFP mice showing that OGT is specifically knocked out in AgRP neurons.
(F) Real-time PCR (F) and western blotting (G) show that OGT is not ectopically deleted in other metabolic tissues.
Figure S3. Metabolic Phenotypes of AgRP-Ogt Knockout Mice, Related to Figure 5
(A) Expression of Ucp1 protein in rWAT of AgRP-Ogt mice treated with saline or ghrelin for 1h. Densitometric analysis is shown to the right.
(B) Body weight of 5-month-old mice (male, n = 17-20; female, n = 9-10).
(C) Daily food intake in 2-month-old female mice (n = 5-6).
(D) Expression of Agrp and Npy transcripts in the hypothalamus (n = 3-4).
(E) Expression of OGT in AgRP neurons of Npy-hrGFP mice at room temperature (RT) or after 24 hr of 4°C exposure.
(F) Core body temperature of CT and KO mice during cold challenge (n = 4-6).
(G) Serum insulin levels after overnight fasting (n = 4-8).
(H) Insulin tolerance test in 5-month-old female mice (n = 4-7). Data are shown as mean ± SEM. *p < 0.05; **p < 0.01; and ***p < 0.001 by unpaired Student’s t test.
Figure S4. Phenotypes of AgRP-Ogt Male Mice Fed on HFD, Related to Figure 7

(A) Growth curve of male mice fed HFD (n = 12-17).

(B) Fat mass of 5-month-old male HFD mice (n = 12-17).

(C–E) Fasting blood glucose (C), fasting serum insulin (D), and HOMA-IR (E) in 6-month-old male HFD mice (n = 11-15).

(F) Insulin tolerance test of 5-month-old male HFD mice. Area under curve (AUC) is shown to the right (n = 6-9). Data are shown as mean ± SEM. *p < 0.05 and **p < 0.01 by unpaired Student's t test.