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Neuronal Deletion of Ghrelin Receptor Almost Completely Prevents Diet-Induced Obesity

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Ghrelin signaling has major effects on energy and glucose homeostasis, but it is unknown whether ghrelin's functions are centrally and/or peripherally mediated. The ghrelin receptor, growth hormone secretagogue receptor (GHS-R), is highly expressed in the brain and detectable in some peripheral tissues. To understand the roles of neuronal GHS-R, we generated a mouse line where *Ghsr* gene is deleted in all neurons using synapsin 1 (Syn1)-Cre driver. Our data showed that neuronal *Ghsr* deletion abolishes ghrelin-induced spontaneous food intake but has no effect on total energy intake. Remarkably, neuronal *Ghsr* deletion almost completely prevented diet-induced obesity (DIO) and significantly improved insulin sensitivity. The neuronal *Ghsr*-deleted mice also showed improved metabolic flexibility, indicative of better adaption to different fuels. In addition, gene expression analysis suggested that hypothalamus and/or midbrain might be the sites that mediate the effects of GHS-R in thermogenesis and physical activity, respectively. Collectively, our results indicate that neuronal GHS-R is a crucial regulator of energy metabolism and a key mediator of DIO. Neuronal *Ghsr* deletion protects against DIO by regulating energy expenditure, not by energy intake. These novel findings suggest that suppressing central ghrelin signaling may serve as a unique antiobesity strategy.

Ghrelin is the only known orexigenic hormone that stimulates food intake and promotes obesity and insulin resistance (1,2). Ghrelin's effects are mediated through its receptor, growth hormone secretagogue receptor (GHS-R) (3). GHS-R is primarily expressed in the brain, with highest expression detected in the hypothalamus, and lower expression is detected in other brain regions and some peripheral tissues (4–6). Ample literature is on ghrelin's multifaceted roles, but the sites of action of GHS-R are unclear. Zigman et al. (7) reported that *Ghsr*-null mice are resistant to diet-induced obesity (DIO), and we reported that *Ghsr*-null mice have reduced glucose under fasting and calorie restriction (8). We also reported that global *Ghsr* ablation alleviates adiposity and insulin resistance during aging by enhancing thermogenesis (9). These findings indicate that ghrelin signaling has important roles in energy and glucose homeostasis. However, since all these findings were obtained from global *Ghsr*-null mice, it is difficult to determine whether these effects are centrally or peripherally mediated. In the current study, we generated neuronal *Ghsr*-deleted mice (*Syn1-Cre*;*Ghsr*$^{f/f}$) to assess the functions of GHS-R in the neurons.

RESEARCH DESIGN AND METHODS

Animals

GHS-R floxed (*Ghsr*$^{f/f}$) mice were originally obtained from Taconic Farms (10). We modified, backcrossed, and then bred them with synapsin 1 (Syn1)-Cre mice (11) to generate *Syn1-Cre*;*Ghsr*$^{f/f}$ mice. Age-matched male *Ghsr*$^{f/f}$ and *Syn1-Cre*;*Ghsr*$^{f/f}$ mice were fed either regular diet (RD) or high-fat diet (HFD). The HFD used is a Western diet from Harlan Teklad (Madison, WI), TD.88137, which has been shown to mimic metabolic syndrome better than other

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HFDs (12). Mice were housed at ~23 ± 1°C with 12-h light/dark cycles. All experimental procedures were approved by the institutional animal care and use committee of Baylor College of Medicine.

Indirect Calorimetry, Glucose Tolerance Test, and Insulin Tolerance Test

Indirect calorimetry data were obtained using an Oxymax system (Columbus Instruments, Columbus, OH), as we previously described (9). For glucose tolerance test (GTT), mice were fasted overnight, and D-glucose (2.0 g/kg) was intraperitoneally injected. For insulin tolerance test (ITT), mice were fasted for 6 h starting at 8:00 A.M., and Humulin (1 unit/kg) was intraperitoneally injected.

Tissue Collection

All tissues were collected immediately after 4 h of 4°C cold exposure. The whole brains were kept on dry ice. Subsequently, each brain region was dissected by the Palkovits punch technique (13). In brief, the brain was sectioned into 200-μm-thick coronal sections using a freezing microtome, with the plane of section adjusted to the Mouse Brain atlas (14). Sections were then mounted on frozen microscope slides, and individual regions were rapidly micropunched under a magni-focuser (Edroy Products Company, Inc., Nyack, NY) using 21 or 19 G Neuro Punches (Fine Science Tools, Inc., Foster City, CA). Punches were blown out of the needles in a chilled tube and then immediately frozen on dry ice.

Quantitative Real-Time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) or RNeasy Mini kit (Qiagen, Germantown, MD). The *Ghsr1a* primers used were as follows: forward primer 5′-GGACCAGAACCACAAACAGACA-3′ and reverse primer 5′-CAGCAGAGGATGAAAGCAAACA-3′ (6). This primer set flanks the intron, which distinguishes *Ghsr1a* from *Ghsr1b*. The rest of the primer information is provided in Supplementary Table 1.

Ghrelin-Induced Spontaneous Food Intake

Ghrelin's effect on spontaneous food intake was conducted as we previously reported (3). After a 3-h fast (7:00 to 10:00 A.M.), mice were intraperitoneally injected with physiologic saline, and then food intake was measured. After 30 min, the same mice were intraperitoneally injected with ghrelin at 0.5 mg/kg body weight.

Cold Challenge Study

Core body temperature was measured in mice using a TH-8 Thermalert monitoring thermometer (Physitemp Instruments, Inc., Clifton, NJ). Mice were individually caged for 4 h at 4°C. Body temperature was assessed hourly, and mice were then immediately sacrificed.

Statistical Analysis

GraphPad Prism was used, and data were represented as mean ± SEM. Two-way ANOVA (with or without repeated measures) or one-way ANOVA was used and followed by Sidak post hoc test. $P < 0.05$ was considered statistically significant. For experiments involving multiple comparisons (Fig. 4), Bonferroni correction was used. The adjusted P value was calculated as α (0.05) divided by the number of genes tested.

RESULTS

Generation of Neuron-Specific *Ghsr*-Deleted Mice

First, the FRT-PGK-neo-FRT cassette was removed from the original $Ghsr^{f/f}$ mice (10) and then backcrossed onto the C57BL background for 10 generations. *Syn1-Cre*;$Ghsr^{f/f}$ were generated by breeding $Ghsr^{f/f}$ mice with *Syn1-Cre* mice (11), as shown in Fig. 1*A*. *Ghsr* expression in *Syn1-Cre*;$Ghsr^{f/f}$ mice was significantly decreased in the brain, but not in peripheral tissues (Fig. 1*B*), indicating that *Ghsr* deletion in *Syn1-Cre*;$Ghsr^{f/f}$ mice is neuron specific. Ghrelin is involved in meal initiation, and ghrelin administration stimulates appetite (15). Indeed, we found that ghrelin-induced acute food intake was abolished in *Syn1-Cre*;$Ghsr^{f/f}$ mice (Fig. 1*C*), supporting that neuronal GHS-R is indispensable for spontaneous food intake. Ghrelin is also known to stimulate growth hormone and IGF1, but we did not detect significant differences in body length or IGF1 levels (Supplementary Fig. 1).

Neuron-Specific *Ghsr* Deletion Largely Prevents DIO and Improves Insulin Sensitivity

Under RD feeding, the body weights of *Syn1-Cre;*$Ghsr^{f/f}$ mice were lower than $Ghsr^{f/f}$ mice starting from 22 weeks of age (Fig. 1*D*), but changes in body fat (Fig. 1*E*) and lean mass (Supplementary Fig. 2A) failed to reach statistical significance. To determine whether neuronal GHS-R regulates glucose homeostasis and insulin sensitivity, we assessed fasting blood glucose and insulin levels and performed ITT and GTT. Although RD-fed *Syn1-Cre*;$Ghsr^{f/f}$ mice showed normal glucose after overnight fasting, they exhibited lower fasting insulin (Fig. 1*F*). The ITT and GTT were not different at either 22 or 44 weeks of age (Supplementary Fig. 3). Next, we studied the mice under DIO by feeding them (HFD starting at 10 weeks of age). Remarkably, *Syn1-Cre*;$Ghsr^{f/f}$ mice showed significantly lower body weight and body fat at as early as 14 weeks of age (after only 4 weeks of HFD feeding), maintaining body weight comparable to that of RD-fed mice (Fig. 1*G* and *H*). These data show that *Syn1-Cre*;$Ghsr^{f/f}$ mice are almost completely resistant to DIO. In addition, HFD-fed *Syn1-Cre*;$Ghsr^{f/f}$ mice showed lower fasting plasma glucose and insulin (Fig. 1*I*). Moreover, lower glucose was detected during ITT, and markedly reduced insulin was detected during GTT (Fig. 1*J* and *K*). These results demonstrate that neuronal deletion of *Ghsr* effectively mitigates DIO-induced insulin resistance.

Neuronal-Specific *Ghsr* Deletion Improves Metabolic Flexibility

Metabolic flexibility is used to describe the body's ability to switch back and forth between major energy sources of carbohydrate (glucose) and fat, according to availability and needs. Metabolic flexibility indicates how rapidly

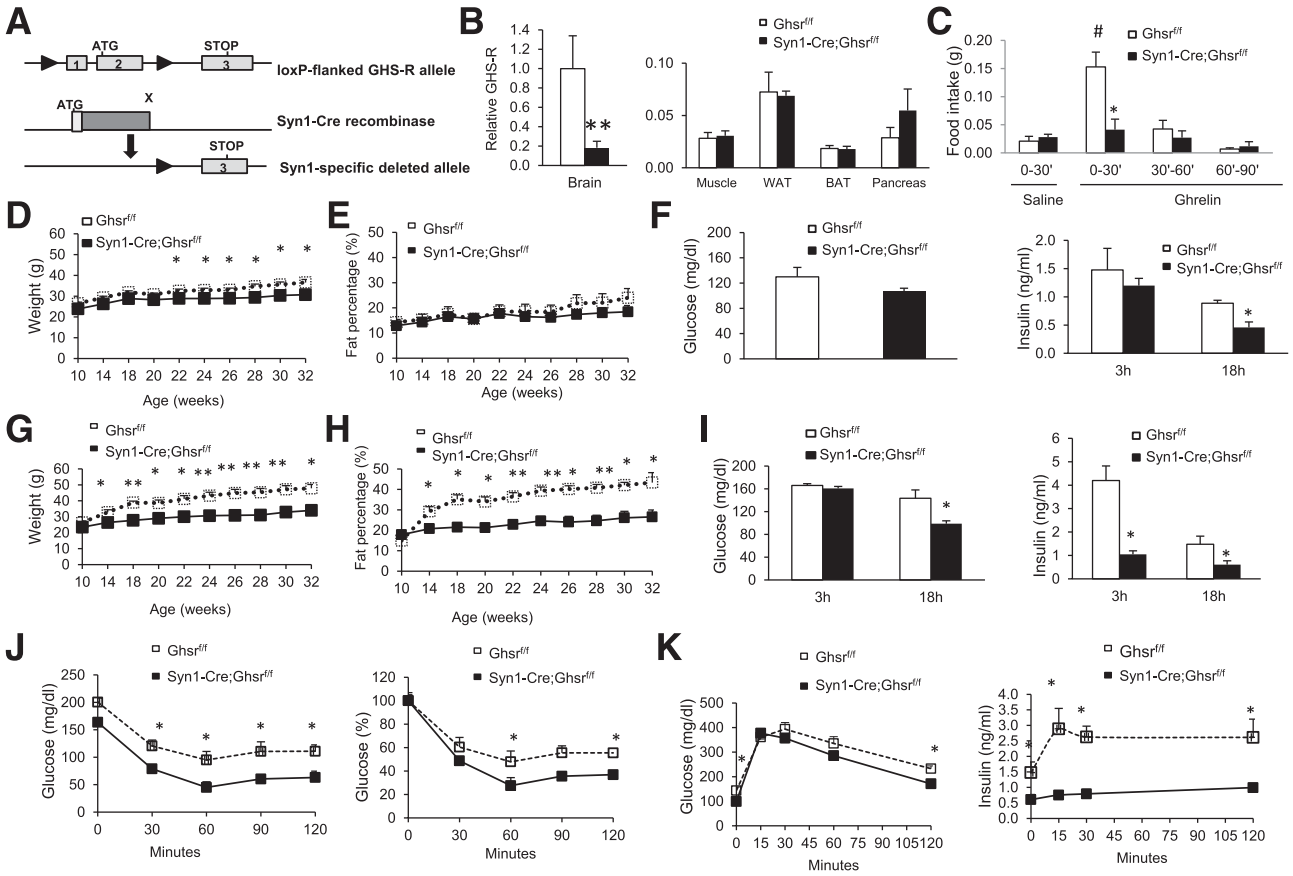

Figure 1—GHS-R ablation in neurons nearly completely prevents DIO and improves insulin sensitivity. *A*: Schematic diagram of the loxP-flanked *Ghsr* allele before and after Cre-derived recombination. Exons 1 and 2 are deleted during recombination. Triangle represents loxP sites. *B*: *Ghsr* gene expression in whole brain, skeletal muscle, epididymal white adipose tissue (WAT), BAT, and pancreas. *C*: Ghrelin-induced acute food intake. Ghrelin (0.5 mg/kg) was intraperitoneally injected into mice in the early morning after 3 h fasting. #$P < 0.05$, saline vs. 30 min after ghrelin injection in $Ghsr^{f/f}$ mice; *$P < 0.05$, $Ghsr^{f/f}$ vs. Syn1-*Cre*;$Ghsr^{f/f}$. *D* and *E*: Body weight and fat percentage of $Ghsr^{f/f}$ and *Syn1-Cre*;$Ghsr^{f/f}$ mice fed RD. *F*: Blood glucose of RD-fed mice after 18 h (overnight) fasting and insulin levels after 3 and 18 h fasting. *G* and *H*: Body weight and fat percentage of mice fed HFD. *I*: Blood glucose and insulin levels of HFD-fed mice after 3 and 18 h fasting. *J*: ITT of 24-week-old HFD-fed mice after 6 h fasting. $P < 0.05$ for interaction between time and genotype for glucose. *K*: Glucose and insulin levels during GTT of 20-week-old HFD-fed mice after 18 h fasting. $P < 0.05$ for interaction between time and genotype for glucose or insulin. n = 6–7. *$P < 0.05$ and **$P < 0.001$, $Ghsr^{f/f}$ vs. *Syn1-Cre*;$Ghsr^{f/f}$. All data are presented as means ± SEM.

animals adapt to dietary changes and how fast they refeed after fasting (16). Ghrelin restores hunger and energy expenditure profiles in metabolically inflexible obese subjects, but not in metabolically flexible postgastrectomy patients (17). To assess whether neuronal-specific *Ghsr* deletion affects metabolic flexibility, we studied respiratory exchange rate (RER) of RD-fed young mice that were fasted for 24 h and then switched to HFD. Interestingly, *Syn1-Cre*;$Ghsr^{f/f}$ mice had increased RER under RD but decreased RER after switching to HFD (Fig. 2*A*). The fasting-induced rebound HFD feeding in young *Syn1-Cre*;$Ghsr^{f/f}$ mice showed a trend of increase within 2 h (Fig. 2*B*). We subsequently studied fasting-induced rebound feeding in mice that had been fed for 32 weeks on either RD or HFD. *Syn1-Cre*;$Ghsr^{f/f}$ mice showed more pronounced rebound feeding under HFD (Fig. 2*D*) but not RD (Fig. 2*C*). These results suggest that *Syn1-Cre*;$Ghsr^{f/f}$ mice have better metabolic flexibility, and neuronal GHS-R has more impact on metabolic flexibility under obese conditions.

Metabolic Characterization of Neuron-Specific *Ghsr*-Deleted Mice

To further investigate the metabolic effect of neuronal *Ghsr* deletion, we conducted indirect calorimetry analysis. Neuronal *Ghsr* deletion showed slightly decreased energy intake and significantly reduced expenditure under RD feeding (Supplementary Fig. 2*B*–*E*). In contrast to RD feeding, there was no difference in total daily HFD intake (Fig. 3*A*). Intriguingly, HFD-fed *Syn1-Cre*;$Ghsr^{f/f}$ mice showed significantly increased energy expenditure (Fig. 3*B* and *C*) and locomotor activity (Fig. 3*D*), but no difference in resting metabolic rate (Fig. 3*E*). Active ghrelin plasma was significantly increased in HFD-fed *Syn1-Cre*;$Ghsr^{f/f}$ mice (Fig. 3*F*), indicative of a compensatory upregulation of ghrelin. Together the data suggest that the

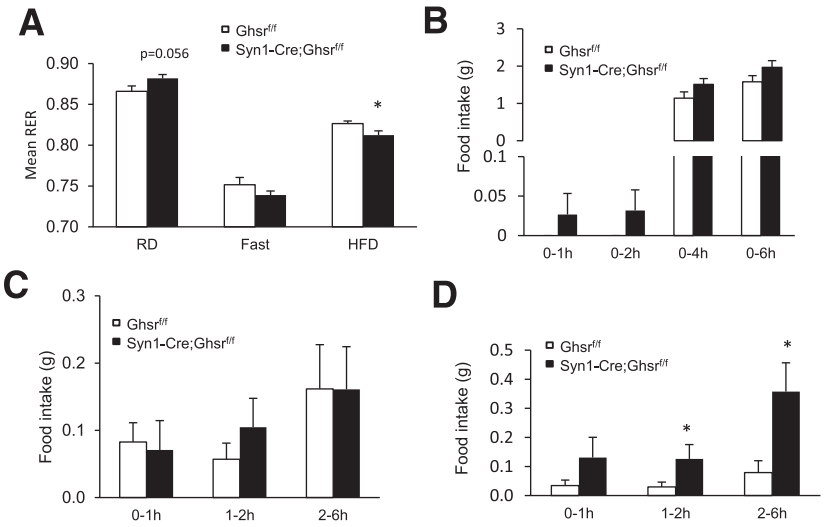

Figure 2—Neuron-specific GHS-R deletion improves metabolic flexibility. *A*: Mean RER under RD, fasting, and HFD refeeding. RD-fed 9-week-old mice were studied in metabolic cages. The mice were fasted for 24 h and then switched to HFD. The RER data were extracted from the 24-h period before and after fasting. *B*: HFD refeeding after 24-h fast. Fasting-induced rebound feeding of 32-week-old RD- or HFD-fed mice: food intake of RD-fed mice during the first 6 h of refeeding after overnight fasting (*C*), and food intake of HFD-fed mice during the first 6 h of refeeding after overnight fasting (*D*). n = 6–10. *$P < 0.05$, *Ghsr*$^{f/f}$ vs. *Syn1-Cre*;*Ghsrf*$^{/f}$. All data are presented as means ± SEM.

lean and insulin-sensitive phenotype of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice was not caused by reduced energy intake but by increased energy expenditure.

Neuron-Specific *Ghsr* Deletion Increases Physical Activity Under DIO

To further assess the effect of GHS-R on voluntary physical activity, the mice were subjected to the running wheel test after 32 weeks of HFD feeding. The long-term HFD feeding completely suppressed the wheel-running activity of *Ghsr*$^{f/f}$ mice (Fig. 3*G* and *H*). Remarkably, HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice remained extremely active, and their wheel running activity showed much greater daily running distance (Fig. 3*I*). HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice preserved wheel-running capacity comparable to that of RD-fed lean mice (Supplementary Fig. 4); moreover, HFD-fed *Syn1*-Cre;*Ghsr*$^{f/f}$ mice also showed a slight increase in locomotor activity (Fig. 3*D*). In contrast, RD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice showed modestly increased voluntary running but decreased locomotor activity (Supplementary Fig. 4). These data suggest that the lean and insulin-sensitive phenotype of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice is, at least in part, due to increased voluntary and spontaneous physical activity.

Neuron-Specific *Ghsr* Deletion Enhances Nonshivering Thermogenesis

To assess the effect of GHS-R on thermogenesis, we subjected the HFD-fed mice to 4°C cold exposure for 4 h. Strikingly, HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice were much more cold resistant, showing higher core body temperature (Fig. 3*K*). Brown adipocytes in brown adipose tissue (BAT) and "beige" adipocytes in subcutaneous fat possess thermogenic properties (18,19). The weight ratio of BAT to total body weight of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice showed no difference (Fig. 3*L*, inset). Consistent with the lean phenotype, the weight ratio of inguinal fat to total body weight was decreased (Fig. 3*M*, inset). We detected increased expression of thermogenic regulatory genes such as uncoupling protein 1 (UCP1), UCP3, PPARγ coactivator 1-α (PGC1α), and β3-adrenergic receptor (β3-AR) in both BAT (Fig. 3*L*) and inguinal fat (Fig. 3*M*). Similarly, RD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice exhibited higher core body temperature and increased thermogenic and beige gene expression in subcutaneous fat (Supplementary Fig. 5). These results suggest that the lean and insulin-sensitive phenotype of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice is contributable to the increased thermogenesis as well.

Hypothalamic GHS-R Expression Is Responsive to DIO

Arcuate nucleus (ARC) and ventromedial hypothalamus (VMH) are known targets of ghrelin. To understand why the mice show more pronounced metabolic phenotype under HFD but not RD feeding, we compared *Ghsr* expression in microdissected hypothalamic ARC and VMH. GHS-R expression was increased in ARC and VHM of HFD-fed mice but not in that of RD-fed mice (Fig. 4*A*). The data suggest that GHS-R signaling is upregulated under DIO, which is in line with the observation that the metabolic phenotype of *Syn1-Cre;Ghsr*$^{f/f}$ mice is more pronounced under DIO.

Syn1-Cre–Driven *Ghsr* Deletion in Various Brain Regions

Syn1-Cre mice have been widely used to generate conditional deletion of neurons (11). To investigate the sites of GHS-R actions, we microdissected the following brain regions from HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ and *Ghsr*$^{f/f}$ mice: ARC, VMH, paraventricular nucleus (PVN), lateral hypothalamus (LH),

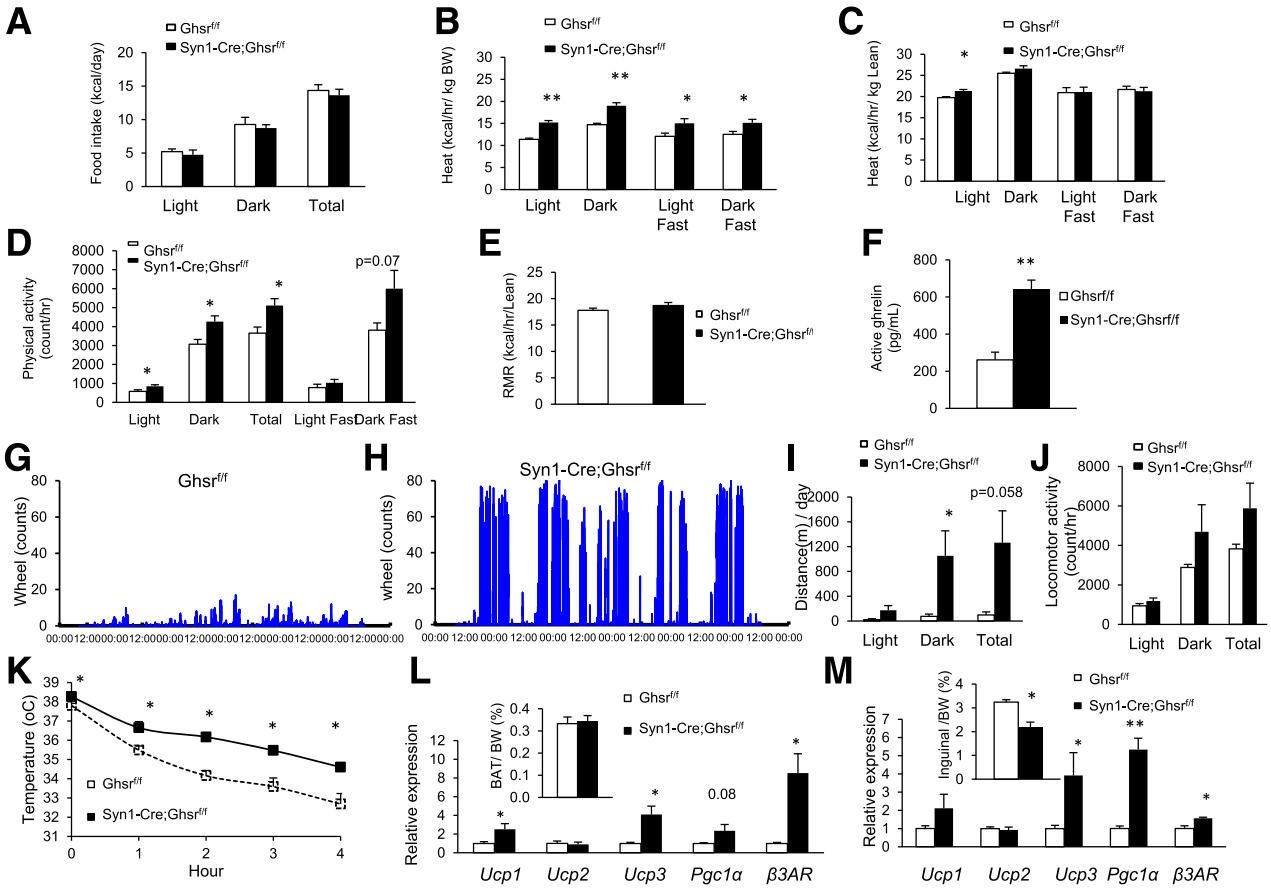

Figure 3— Neuronal GHS-R ablation increases energy expenditure, exhibiting increased physical activity and thermogenesis. *A–D*: Indirect calorimetry analysis of 18-week-old mice. *A*: Daily food intake. *B* and *C*: Energy expenditure (heat) adjusted by body weight or lean mass. *D*: Locomotor activity. *E*: Resting metabolic rate (RMR) was measured during light cycle and normalized by lean mass. *F*: Plasma active ghrelin of mice fed HFD for 32 weeks, after 3 h of morning fasting. n = 6–7. Metabolic profile of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice with running wheels: 5-day recording of wheel rotations of *Ghsr*$^{f/f}$ mice and *Syn1-Cre*;*Ghsr*$^{f/f}$ mice (*G* and *H*), average daily running distance (*I*), and locomotor activity with running wheels (*J*). *K*: Rectal temperature of HFD-fed mice during 4°C cold exposure. $P < 0.05$ for interaction between time and genotype for temperature. *L*: The weight ratio of BAT to total body weight (BW), and expression of thermogenic regulatory genes in BAT. *M*: The weight ratio of subcutaneous (inguinal) fat to total BW and expression of thermogenic regulatory genes in subcutaneous fat of HFD-fed mice. Tissues were collected immediately after cold challenge for 4 h at 4°C. n = 6–7. *$P < 0.05$ and **$P < 0.001$, *Ghsr*$^{f/f}$ vs. *Syn1-Cre*;*Ghsrf*$^{/f}$. All data are presented as means ± SEM.

ventral tegmental area (VTA), substantia nigra (SN), and locus coeruleus (LC). *Ghsr* expression was reduced by 50% in ARC and VMH, by 75% in PVN, and by 80–90% in VTA and SN, with no change in LH (Fig. 4*B*). Our data indicate that synapsin-Cre is not activated identically in all neurons. In ARC, in line with the modest *Ghsr* deletion, expression of orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP), as well as cannabinoid receptor type 1 (CB1), showed only a slight trend of decrease (not statistically significant), whereas anorexic pro-opiomelanocortin (POMC) was unchanged in *Syn1-Cre*;*Ghsr*$^{f/f}$ mice (Fig. 4*C*).

Putative Sites for Neuronal GHS-R–Mediated Thermogenic Regulation

VMH is an important site for the regulation of energy expenditure, involving regulatory pathways such as steroidogenic factor-1 (SF1), CB1-mediated leptin signaling, and AMPK-sympathetic nervous system-BAT axis-mediated thermogenic signaling (16,20). It has been shown that AMPK mediates ghrelin's effects in several tissues (1,2). Interestingly, AMPK1α expression was decreased in VMH of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice (Fig. 4*D*). Leptin is an anorexic hormone, and leptin regulates thermogenesis via VMH. However, expression of leptin receptor (LepR) and its downstream mediator STAT3 in VMH was not changed (Fig. 4*D*). PVN is also important in the control of feeding and energy expenditure (21). NPY in ARC inhibits thermogenesis via downregulation of tyrosine hydroxylase (TH) in the PVN (22); CB1 in PVN antagonizes leptin signaling to reduce thermogenesis (23). Indeed, TH expression in *Syn1-Cre*;*Ghsr*$^{f/f}$ mice was increased in PVN, whereas CB1 expression was decreased in PVN (Fig. 4*E*). Orexin neurons in LH have widespread projections that modulate BAT thermogenesis (24). Orexin was significantly increased in LH of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice (Fig. 4*F*). LC is part of a thermoeffector neuronal

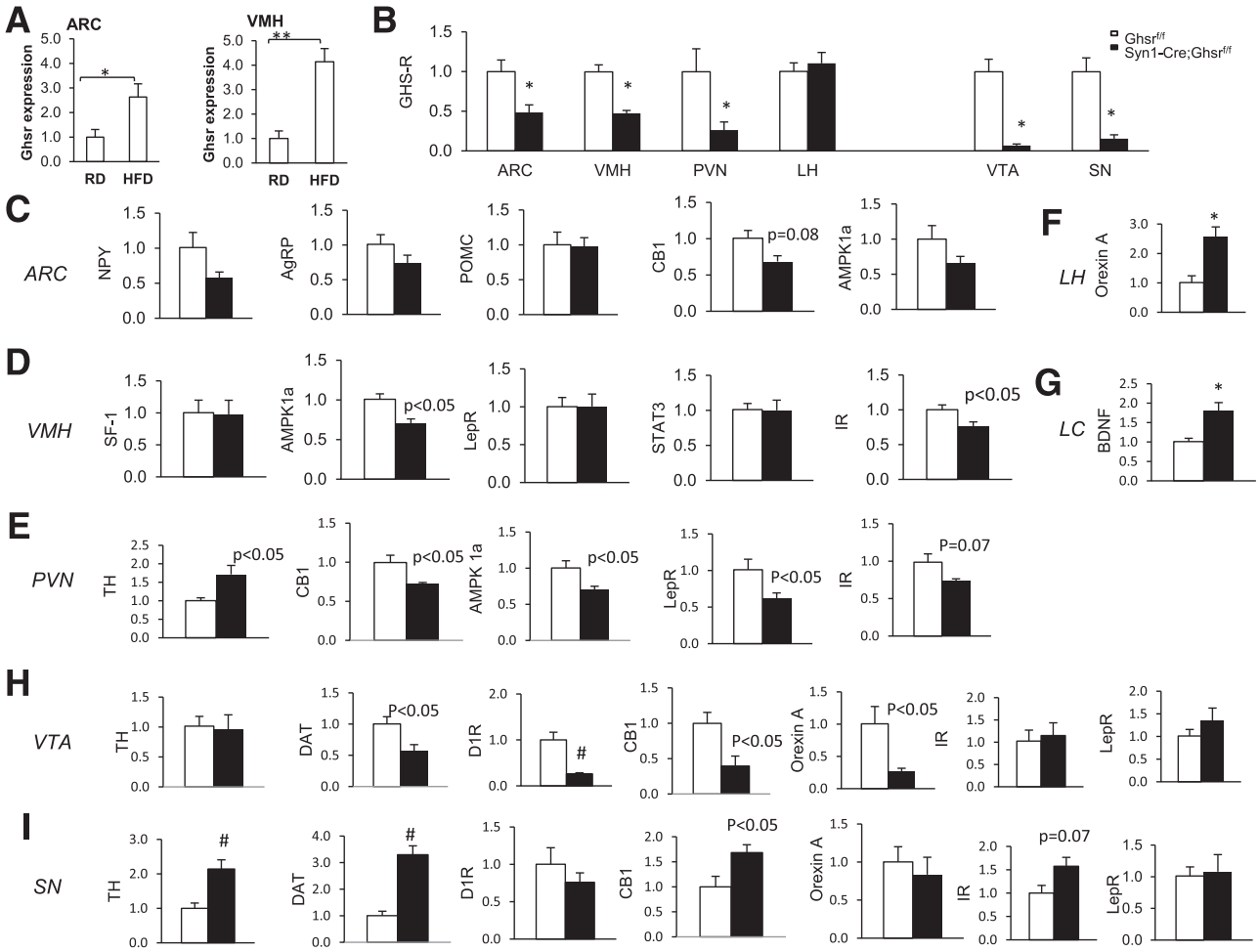

Figure 4—Neuronal GHS-R ablation activates thermogenic signaling pathways in hypothalamic regions and enhances dopaminergic regulatory genes in midbrain. GHS-R expression in ARC and VMH of wild-type mice fed either RD or HFD (*A*), and in various brain regions of *Ghsr*$^{f/f}$ and *Syn1-Cre*;*Ghsrf*$^{/f}$ mice (*B*). Expression of putative regulators associated with GHS-R signaling in ARC (*C*), VMH (*D*), PVN (*E*), LH (*F*), LC (*G*), VTA (*H*), and SN (*I*) of HFD-fed *Syn1-Cre*;*Ghsrf*$^{/f}$ mice. All data are presented as means ± SEM. n = 6–7. *P < 0.05 and **P < 0.001, *Ghsr*$^{f/f}$ vs. *Syn1-Cre*;*Ghsrf*$^{/f}$. For gene expression in different brain regions, Bonferroni correction was applied. #P < 0.007, considered as statistically significant for VAT and SN.

pathway that contains major noradrenergic nuclei (25), and brain-derived neurotrophic factor (BDNF) is critical in maintaining plasticity of neurons and synapses (26). BDNF was increased in LC of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice (Fig. 4*G*), suggesting improved neuronal plasticity and functions. It is also reported that VTA is associated with thermogenesis (27); CB1 and orexin A are mediators of ghrelin's orexigenic function (28). Our data showed that whereas CB1 and orexin A were decreased in VTA (Fig. 4*H*), CB1 was increased in SN (Fig. 4*I*). These data suggest that increased thermogenic activity of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice is likely associated with the functions of GHS-R in hypothalamic neurons.

Putative Sites for Neuronal GHS-R–Mediated Physical Activity Regulation

The neurotransmitter dopamine is a regulator of locomotor activity and feeding behavior (29), and dopamine signaling is impaired in obesity (30–32). Whereas dopaminergic VTA neurons are primarily involved in reward and motivation (33), SN neurons are primarily involved in regulation of spontaneous movement and active physical activity (33). In Parkinson disease, the loss of dopaminergic neurons in SN results in progressive motor deficits (34). GHS-R forms heterodimers with dopamine D1-like receptor (D1R) to amplify dopamine signaling (35,36) and with D2R to regulate food intake (35,37). To access dopaminergic activation in *Syn1-Cre*;*Ghsr*$^{f/f}$ mice, the dopamine synthesis enzyme TH and dopamine uptake regulator dopamine active transporter (DAT), as well as dopamine receptors D1R and D2R, were studied. As expected, D1R and D2R expression was suppressed by long-term HFD feeding, and D2R was not detectable under HFD feeding. Intriguingly, TH was not changed in VTA of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice but increased in their SN. DAT and CB1 were decreased in VTA neurons of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice but increased in

their SN neurons (Fig. 4*H* and *I*). These results suggest that GHS-R has differential effects in VTA and SN neurons. *Ghsr* deletion in VTA may reduce dopamine reuptake, thus suppressing dopamine turnover and reducing reward- and motivation-associated responses. *Ghsr* deletion in SN neurons may increase dopamine synthesis and dopamine reuptake, leading to enhanced dopamine activity. The elevated physical activity of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice possibly involves the actions of GHS-R in SN, and GHS-R may have an important role in fine-tuning dopaminergic activity.

DISCUSSION

We and others have shown that global deletion of *Ghsr* in mice attenuates DIO and improves insulin sensitivity (7,9,38,39). Our new *Syn1-Cre*;*Ghsr*$^{f/f}$ mice in the current study enable us to investigate the effects of GHS-R in neurons, independent from its effects in peripheral tissues. Our finding indicates that neuronal GHS-R is essential for ghrelin-induced meal initiation but is not required for long-term food intake. Neuron-specific deletion of *Ghsr* completely prevents DIO and significantly improves DIO-induced insulin resistance, exhibiting activated thermogenesis and enhanced physical activity. Our data demonstrate for the first time that central ghrelin signaling regulates long-term energy homeostasis not by its effect on total energy intake but by its effects on energy expenditure driven by centrally mediated thermogenesis and physical activity.

RD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice exhibit reduced food intake and energy expenditure, which is different from that of global *Ghsr*-null mice. Our data show that the difference in energy expenditure becomes obscure when the mice were pair fed (Supplementary Fig. 6), suggesting that the reduced energy expenditure observed in RD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice may be a complementary response to reduced food intake. Our studies of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice reveal that neuronal GHS-R mediates ghrelin-induced acute orexigenic effects and DIO-induced adiposity and insulin resistance but has no effect on HFD intake. Similar to our finding, Wang et al. (40) recently reported that AgRP neuron-selective *Ghsr* re-expression partially restores ghrelin-induced acute food intake but has no effect on daily food intake. These results challenge the dogmatic view that ghrelin signaling controls energy balance by its orexigenic effect in AgRP neurons and suggest that neurons other than AgRP are important for mediating the metabolic effects of GHS-R. Our data also revealed that *Syn1-Cre*;*Ghsr*$^{f/f}$ mice have improved metabolic flexibility under DIO, using carbohydrate as an energy source under RD and using fat as an energy source under HFD. HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice refed faster after fasting, which also indicates improved metabolic flexibility. These observations together demonstrate that *Syn1-Cre*;*Ghsr*$^{f/f}$ mice can quickly adapt to the diet they are given and use the available energy efficiently.

Our data further demonstrate that neuronal deletion of *Ghsr* almost completely prevents DIO, showing much more robust resistance to DIO than that observed in global *Ghsr*-null mice (7). Moreover, neuronal deletion of *Ghsr* also shows significant attenuation of DIO-induced insulin resistance. These novel data underscore the importance of neuronal GHS-R in energy homeostasis. Our metabolic analysis reveals that *Syn1-Cre*;*Ghsr*$^{f/f}$ mice are protected against DIO by increased energy expenditure, specifically by increased thermogenesis and physical activity. In global *Ghsr*-null mice, we only observed increased thermogenesis but not increased physical activity (9,38). The differential physical phenotypes exhibited by *Syn1-Cre*;*Ghsr*$^{f/f}$ and *Ghsr*$^{-/-}$ mice suggest that neuronal GHS-R plays a central role in regulation of physical activity, and peripherally expressed GHS-R may have opposing or compensatory effects on physical activity. In addition, our data show that HFD increases *Ghsr* expression in hypothalamic nuclei such as ARC and VMH, which is in agreement with our observation that the effects of neuronal GHS-R were much more pronounced under HFD feeding than RD feeding. These new evidences suggest that neuronal GHS-R signaling is activated primarily under obese and insulin-resistant conditions and may serve as a more specific and selective antiobesity target.

In the current study, *Ghsr* expression in different brain regions was further assessed. Our data indicate that although Syn1-Cre has the ability to target all neurons, the efficiency varies by region. Our gene expression data shed new light on the potential sites that may mediate the effects of GHS-R on physical activity and thermogenesis.

Physical activity includes spontaneous and voluntary activities. Spontaneous activity is referred to as obligatory activities such as eating and grooming; voluntary activity is referred to as self-motivated movements such as running and other intensive undertakings. Our results demonstrate that neuronal *Ghsr* deletion promotes both spontaneous and voluntary activity. Our data suggest that neuronal GHS-R may play a permissive role for physical activity under DIO; elevated energy expenditure exhibited by HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice may be, at least in part, attributable to increased physical activity. DAT is a known homeostatic regulator of dopamine tone under normal physiological conditions (41). In obesity-prone rats, DAT expression is significantly reduced compared with that of obesity-resistant rats (30), suggesting that the DAT function is important for DIO. The loss of dopaminergic neurons in SN is recognized as the underlying mechanism associated with loss of voluntary movement in Parkinson disease (34). Central ghrelin administration has been reported to decrease spontaneous locomotor activity in rats (42), and ghrelin-deficient mice show increased physical activity (43). In contrast, another report showed that ghrelin administration into VTA induces locomotor activity in mice (44). The discrepancy may be due to differences in experimental conditions. It has been shown that ghrelin has differential effects on dopaminergic activity in midbrain SN and VTA (35,45,46). In agreement with the

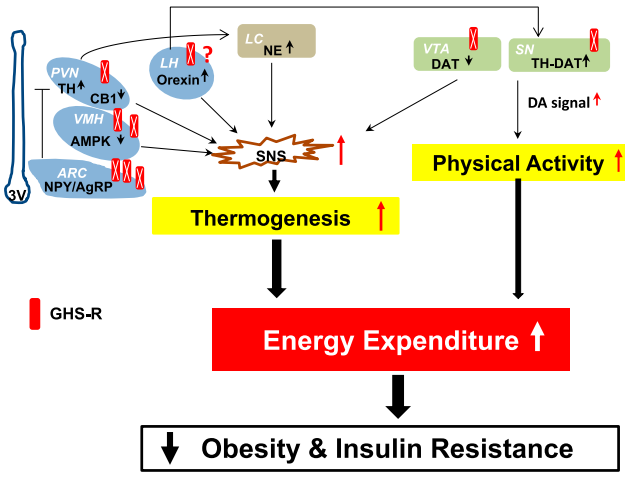

Figure 5—Schematic diagram of proposed actions of neuronal GHS-R on thermogenesis and physical activity. Our data suggest that ghrelin signaling may centrally regulate thermogenesis and physical activity via the following signaling pathways. *1*) Ablation of GHS-R suppresses NPY in ARC and then stimulates thermogenesis through upregulation of TH in PVN. *2*) Decreased AMPK activation in VMH of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice directly stimulates thermogenesis through sympathetic outflow. *3*) Increased TH or decreased CB1 in PVN of *Syn1-Cre;Ghsr*$^{f/f}$ mice directly stimulates thermogenesis through sympathetic outflow. *4*) Increased orexin in LH of *Syn1-Cre*;*Ghsr*$^{f/f}$ mice directly/indirectly stimulates thermogenesis and enhances physical activity. *5*) Deletion of GHS-R directly increases dopaminergic activity in midbrain SN neurons to enhance physical activity. Taken together, our data suggest that GHS-R regulates energy metabolism by centrally mediated thermogenesis and physical activity. DA, dopamine signal; NE, norepinephrine; SNS, sympathetic nervous system.

literature (47,48), our data show that HFD reduces physical activity and promotes DIO. Higher expression of TH and DAT was detected in SN of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice, which suggests that dopamine turnover and activity might be elevated in the SN; that is in agreement with the increased physical activity observed in HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice. Moreover, our data showed that GHS-R had differential effects in different types of dopaminergic neurons; *Ghsr* deletion appeared to inhibit dopaminergic activity in VTA but increase it in SN. Our data collectively suggest that ghrelin signaling regulates centrally mediated physical activity. Thus, GHS-R may have an important role in fine-tuning dopaminergic activity in midbrain, and studies are needed to further define the role of GHS-R in dopaminergic neurons.

Nonshivering thermogenesis is an important mechanism for maintaining body temperature and burning energy, and obesity is known to be associated with thermogenic impairment (19,49). In rodents, BAT is a major site for thermogenesis. Recent findings indicate that brite/beige adipocytes in subcutaneous adipose tissues are also involved in thermogenesis (18). Our studies showed increased expression of thermogenic regulators UCP1, UCP3, PGC1α, and β3-AR in both BAT and inguinal fat of HFD-fed *Syn1-Cre*;*Ghsr*$^{f/f}$ mice under cold exposure. This is consistent with the thermogenic phenotype we observed in old *Ghsr*-null mice (9). Our data support that neuronal GHS-R has an important role in centrally mediated thermogenesis. Neuronal GHS-R deletion may stimulate central thermogenic signaling to activate thermogenic activity in brown and "beige" adipocytes, thereby increasing thermogenesis. Orexigenic peptide NPY in ARC has been shown to inhibit thermogenesis via downregulation of TH neurons in the PVN (22). At the same time, CB1 signaling in PVN has been shown to antagonize leptin signaling (23). CB1 is a downstream target of GHS-R in NPY/AgRP neurons (50). Orexin neurons are known to regulate food intake and to modulate BAT thermogenesis in LH (24). CB1 and orexin have been suggested to mediate ghrelin's orexigenic effects in VTA (28). Our data showed that *Ghsr* deletion regulates these thermogenic regulatory genes in VMH, PVN, and LH, suggesting that VMH, PVN, and/or LH may be primary sites for GHS-R–mediated thermogenesis.

Our findings unequivocally demonstrate that neuronal GHS-R has a central role in energy metabolism, which is a crucial pathogenic factor of DIO. Neuronal GHS-R deletion increases energy expenditure by modulating centrally mediated physical activity and thermogenesis but not by decreasing total energy intake. Our data further suggest that hypothalamic and dopaminergic neurons may be the sites that mediate the effects of GHS-R on thermogenesis and physical activity, respectively (Fig. 5). Suppressing central ghrelin signaling may serve as a unique antiobesity strategy that can simultaneously enhance physical activity and boost fat burning.

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