Neuronal deletion of ghrelin receptor almost completely prevents diet-induced obesity

Jong Han Lee¹, Ligen Lin¹,², Pingwen Xu¹, Kenji Saito¹, Qiong Wei¹,³, Adelina G. Meadows¹, Odelia Y. N. Bongmba¹, Geetali Pradhan¹, Hui Zheng⁴, Yong Xu¹, & Yuxiang Sun¹,⁴,⁵,⁶*

¹USDA/ARS Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA; ²State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau, China; ³Division of Endocrinology, Zhongda Hospital, Southeast University, Nanjing, Jiangsu, China. ⁴Huffington Center on Aging, Baylor College of Medicine, Houston, TX, USA; ⁵Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA; ⁶Department of Nutrition and Food Science, Texas A&M University, College Station, TX, USA

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*Correspondence to: Yuxiang Sun, Mailing address: Department of Nutrition and Food Science, Texas A&M University, 214D Cater-Mattil; 2253 TAMU, College Station, TX 77843. Phone: 979-862-9143; E-mail: yuxiangs@tamu.edu

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Abstract

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 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-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 anti-obesity 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 hypothalamus, and lower expression 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. reported that Ghsr-null mice are resistant to diet-induced obesity (DIO) (7), 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\textsuperscript{\textminus}) to assess the functions of GHS-R in the neurons.

**Research design and Methods**

**Animals.** GHS-R floxed (Ghsr\textsuperscript{\textplus}) mice were originally obtained from Taconic Farms (10), we modified and backcrossed, then bred them with Synapsin 1 (Syn1)-Cre mice (11) to generate Syn1-Cre;Ghsr\textsuperscript{\textplus} mice. Age-matched male Ghsr\textsuperscript{\textplus} and Syn1-Cre;Ghsr\textsuperscript{\textplus} 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 high fat diet (12). Mice were housed at ~23\pm1°C with 12 h light/dark cycles. All experimental procedures were approved by IACUC of Baylor College of Medicine.
Indirect calorimetry, glucose tolerance test (GTT), and insulin tolerance test (ITT). Indirect calorimetry data were obtained using an Oxymax system (Columbus Instruments, Columbus, OH), as we previously described (9). For GTT, mice were fasted overnight, and D-glucose (2.0 g/kg) was i.p. injected. For ITT, mice were fasted for 6 h starting at 8:00 AM, and Humulin (1 U/kg) was i.p. injected.

Tissue collection. All tissues were collected immediately after 4 hours 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). Briefly, 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 micro-punched under a magni-focuser (Edroy Products Company, Inc., Nyack, NY, USA) using 21 G or 19 G Neuro Punches (Fine Science Tools, Inc., Foster City, CA, USA). Punches were blown out of the needles in a chilled tube, 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). Ghsr1a primers used were: forward primer 5'-GGACCAGAACCACAAACGACA-3', reverse primer 5'-CAGCAGAGGATGAAAGCAAACA-3' (6). This primer set flanks the intron, which distinguish Ghsr1a from Ghsr1b. The rest of the primer information is provided in Supplemental Table 1.

Ghrelin-induced spontaneous food intake. Ghrelin’s effect on spontaneous food intake was conducted as we previously reported (3). After 3 h fast (7:00 to 10:00 A.M), mice were i.p.
injected with physiologic saline, and then food intake was measured. After 30 minutes, the same mice were *i.p.* 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, New Jersey). Mice were individually caged for 4 hours at 4 °C. Body temperature was assessed hourly, and mice were then immediately sacrificed.

**Statistical analysis.** Graph-Pad Prism was used, and data were represented as mean ± SEM. Two-way ANOVA (with or without repeated measures) or One-way ANOVA were used and followed by Sidak’s *post hoc* test. P < 0.05 was considered statistically significant. For experiments involved multiple comparisons (Fig. 4), Bonferroni correction was used. The adjusted p value was calculated as $\alpha (0.05)$ 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*$^{flo}$ mice (10), then backcrossed onto C57BL background for 10 generations. *Syn1-Cre;Ghsr*$^{flo}$ were generated by breeding *Ghsr*$^{flo}$ mice with *Syn1-Cre* mice (11), as shown in **Fig. 1A.** *Ghsr* expression in *Syn1-Cre;Ghsr*$^{flo}$ mice was significantly decreased in the brain, but not in peripheral tissues (**Fig. 1B**), indicating that *Ghsr* deletion in *Syn1-Cre;Ghsr*$^{flo}$ 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*$^{flo}$ mice (**Fig. 1C**), supporting
that neuronal GHS-R is indispensable for spontaneous food intake. Ghrelin is also known to stimulate growth hormone and insulin-like growth hormone (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<sup>f/f</sup> mice were lower than Ghsr<sup>f/f</sup> mice starting from 22-weeks of age (Fig. 1D), but changes in body fat (Fig. 1E) 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. While RD-fed Syn1-Cre;Ghsr<sup>f/f</sup> mice showed normal glucose after overnight fasting, they exhibited lower fasting insulin (Fig. 1F). The ITT and GTT were not different at either 22-weeks 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<sup>f/f</sup> mice showed significantly lower body weight and body fat 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. 1G, 1H). These data show that Syn1-Cre;Ghsr<sup>f/f</sup> mice are almost completely resistant to DIO. In addition, HFD-fed Syn1-Cre;Ghsr<sup>f/f</sup> mice showed lower fasting plasma glucose and insulin (Fig. 1I). Moreover, lower glucose was detected during ITT, and markedly reduced insulin was detected during GTT (Fig. 1J, 1K). 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 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 post-gastrectomy 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 hours and then switched to HFD. Interestingly, Syn1-Cre;Ghsr<sup>ff</sup> mice had increased RER under RD, but decreased RER after switching to HFD (Fig. 2A). The fasting-induced rebound HFD feeding in young Syn1-Cre;Ghsr<sup>ff</sup> mice showed a trend of increase within 2 hours (Fig. 2B). We subsequently studied fasting-induced rebound feeding in mice that had been fed for 32 weeks on either RD or HFD. Syn1-Cre;Ghsr<sup>ff</sup> mice showed more pronounced rebound feeding under HFD (Fig. 2D) but not RD (Fig. 2C). These results suggest that Syn1-Cre;Ghsr<sup>ff</sup> mice have better metabolic flexibility, and neuronal GHS-R has more impact on metabolic flexibility under obese condition.

**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. 2B-2E). Contrast to RD-feeding, there was no difference in total daily HFD intake (Fig. 3A). Intriguingly, HFD-fed Syn1-Cre;Ghsr<sup>ff</sup> mice showed significantly increased energy expenditure (Fig. 3B-3C) and locomotor activity (Fig. 3D), but no difference in resting metabolic rate (Fig. 3E). Active ghrelin
plasma was significantly increased in HFD-fed Syn1-Cre;Ghsr<sup>ff</sup> mice (Fig. 3F), indicative of a compensatory upregulation of ghrelin. Together the data suggest that the lean and insulin-sensitive phenotype of HFD-fed Syn1-Cre;Ghsr<sup>ff</sup> 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<sup>ff</sup> mice (Fig. 3G, 3H). Remarkably, HFD-fed Syn1-Cre;Ghsr<sup>ff</sup> mice remained extremely active, and their wheel running activity showed much greater daily running distance (Fig. 3I). HFD-fed Syn1-Cre;Ghsr<sup>ff</sup> mice preserved wheel-running capacity comparable to that of RD-fed lean mice (Supplementary Fig. 4); moreover, HFD-fed Syn1-Cre;Ghsr<sup>ff</sup> mice also showed a slight increase in locomotor activity (Fig. 3D). In contrast, RD-fed Syn1-Cre;Ghsr<sup>ff</sup> 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<sup>ff</sup> mice is, at least in part, due to increased voluntary and spontaneous physical activity.

**Neuron-specific Ghsr deletion enhances non-shivering thermogenesis**

To assess the effect of GHS-R on thermogenesis, we subjected the HFD-fed mice to 4°C cold exposure for 4 hours. Strikingly, HFD-fed Syn1-Cre;Ghsr<sup>ff</sup> mice were much more cold-resistant, showing higher core body temperature (Fig. 3K). 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 $\text{Syn1-Cre;Ghsr}^{\text{f/f}}$ mice showed no difference (Fig. 3L insert). Consistent with the lean phenotype, the weight ratio of inguinal fat to total body weight was decreased (Fig. 3M insert). We detected increased expression of thermogenic regulatory genes such as uncoupling proteins (UCP)1, UCP3, PPAR$\gamma$ coactivator 1-alpha (PGC1$\alpha$) and $\beta_3$-adrenergic receptor ($\beta_3$-AR) in both BAT (Fig. 3L) and inguinal fat (Fig. 3M). Similarly, RD-fed $\text{Syn1-Cre;Ghsr}^{\text{f/f}}$ 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 $\text{Syn1-Cre;Ghsr}^{\text{f/f}}$ 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 $\text{Ghsr}$ expression in micro-dissected 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. 4A). The data suggest that GHS-R signaling is upregulated under DIO, which is in line with the observation that the metabolic phenotype of $\text{Syn1-Cre;Ghsr}^{\text{f/f}}$ mice is more pronounced under DIO.

**$\text{Syn1-Cre}$ driven $\text{Ghsr}$ deletion in various brain regions**

$\text{Syn1-Cre}$ mice have been widely used to generate conditional deletion of neurons (11). To investigate the sites of GHS-R actions, we micro-dissected the following brain regions from HFD-fed $\text{Syn1-Cre;Ghsr}^{\text{f/f}}$ and $\text{Ghsr}^{\text{f/f}}$ mice: ARC, VMH, paraventricular nucleus (PVN), lateral
hypothalamus (LH), 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. 4B). 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<sup>f/f</sup> mice (Fig. 4C).

**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), cannabinoid receptor type 1 (CB1) mediated leptin signaling, and AMPK-SNS-BAT axis-mediated thermogenic signaling (16, 20). It has been shown that AMPK mediates ghrelin’s effects in several tissues (1, 2). Interestingly, AMPK<sub>1α</sub> expression was decreased in VMH of Syn1-Cre;Ghsr<sup>f/f</sup> mice (Fig. 4D). 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. 4D). PVN is also important in control of feeding and energy expenditure (21). NPY in ARC inhibits thermogenesis via down-regulation 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<sup>f/f</sup> mice was increased in PVN, whereas CB1 expression was decreased in PVN (Fig. 4E). Orexin neurons in LH have widespread projections which modulate BAT thermogenesis (24). Orexin was significantly increased in LH of Syn1-Cre;Ghsr<sup>f/f</sup> mice (Fig. 4F). LC is part of a
thermoeffector neuronal pathway which 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<sup>f/f</sup> mice (Fig. 4G), 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 while CB1 and orexin A were decreased in VTA (Fig. 4H), CB1 was increased in SN (Fig. 4I). These data suggest that increased thermogenic activity of Syn1-Cre;Ghsr<sup>f/f</sup> mice is likely associated with the functions of GHS-R in hypothalamic neurons.

**Putative sites for neuronal GHS-R mediated physical activity regulation**

Neurotransmitter dopamine is a regulator of locomotor activity and feeding behavior (29), and dopamine signaling is impaired in obesity (30-32). While 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’s 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<sup>f/f</sup> mice, dopamine synthesis enzyme tyrosine hydroxylase (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<sup>f/f</sup> mice, but increased in their SN. DAT and CB1 were decreased in VTA neurons of HFD-fed Syn1-Cre;Ghsr<sup>f/f</sup> mice, but increased in their SN neurons (Fig. 4H, 4I). 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<sup>fl/fl</sup> mice possibly involve 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<sup>fl/fl</sup> 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<sup>fl/fl</sup> 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<sup>fl/fl</sup> mice may be a complementary response to reduced food intake. Our studies of Syn1-Cre;Ghsr<sup>fl/fl</sup> 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. recently reported that AgRP neuron-selective Ghsr re-expression partially restores ghrelin-induced acute food intake, but has no effect on daily food intake (40). 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<sup>ff</sup> 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<sup>ff</sup> mice refed faster after fasting, which also indicates improved metabolic flexibility. These observations together demonstrate that Syn1-Cre;Ghsr<sup>ff</sup> 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 underscores the importance of neuronal GHS-R in energy homeostasis. Our metabolic analysis reveals that Syn1-Cre;Ghsr<sup>ff</sup> 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<sup>ff</sup> and Ghsr<sup>-/-</sup> mice suggest that neuronal GHS-R plays an 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 condition, may serve as a more specific and selective anti-obesity target.

In the current study, Ghsr expression in different brain regions was further assessed. Our data indicate that while Synapsin 1 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<sup>+/−</sup> 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 to 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’s 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 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<sup>f/f</sup> 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<sup>f/f</sup> 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, studies are needed to further define the role of GHS-R in dopaminergic neurons.

Non-shivering 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<sup>f/f</sup> 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 down-regulation 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 crucial pathogenic factor of DIO. Neuronal GHS-R deletion increases energy expenditure by modulating centrally-mediated physical activity and thermogenesis, but no 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 anti-obesity strategy that can simultaneously enhance physical activity and boost fat-burning.

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Figure legends

**Fig. 1. GHS-R ablation in neurons nearly completely prevents diet-induced obesity 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), brown adipose tissue (BAT) and pancreas. (C) Ghrelin-induced acute food intake. Ghrelin (0.5 mg/kg) was i.p injected into mice in the early morning after 3 h fasting. # p<0.05, saline vs. 30 min after ghrelin injection in Ghsr<sup>-/-</sup> mice; * p<0.05, Ghsr<sup>-/-</sup> vs. Syn1-Cre;Ghsr<sup>-/-</sup>. (D-E) Body weight and fat percentage of Ghsr<sup>-/-</sup> and Syn1-Cre;Ghsr<sup>-/-</sup> mice fed
regular diet (RD). (F) Blood glucose of RD-fed mice after 18 h (overnight) fasting, and insulin levels after 3 h and 18 h fasting. (G-H) Body weight and fat percentage of mice fed high-fat diet (HFD). (I) Blood glucose and insulin levels of HFD-fed mice after 3 h 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. $n=6-7$. *, $p<0.05$, **, $p<0.001$, $Ghsr^{f/f}$ vs. $Syn1-Cre;Ghsr^{f/f}$. All data are presented as means $\pm$ SEM.

**Fig. 2. Neuron-specific GHS-R deletion improves metabolic flexibility.** (A) Mean Respiratory Exchange Ratio (RER) under RD, fasting, and HFD refeeding. RD-fed 9-week old mice was studied in metabolic cages. The mice were fasted for 24 h then switched to HFD. The RER data were extracted from 24 h period before and after fasting. (B) HFD refeeding after 24 h fast. (C, D) 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); 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. $Syn1-Cre;Ghsr^{f/f}$. All data are presented as means $\pm$ SEM.

**Fig. 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-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
morning fasting. n=6-7. (G-J) Metabolic profile of HFD-fed Syn1-Cre;Ghsr<sup>f/f</sup> mice with running wheels: 5 day recording of wheel rotations of Ghsr<sup>f/f</sup> mice and Syn1-Cre;Ghsr<sup>f/f</sup> mice (G,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, **, p<0.001 Ghsr<sup>f/f</sup> vs Syn1-Cre;Ghsr<sup>f</sup>. All data are presented as means ± SEM.

**Fig. 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 WT mice fed either RD or HFD (A), and in various brain regions of Ghsr<sup>f/f</sup> and Syn1-Cre;Ghsr<sup>f</sup> 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;Ghsr<sup>f</sup> mice. All data are presented as means ± SEM. n=6-7. *, p<0.05, **, p<0.001 Ghsr<sup>f/f</sup> vs Syn1-Cre;Ghsr<sup>f</sup>. For gene expression in different brain regions, Bonferroni correction was applied. †, p<0.007 is considered as statistically significant for VAT and SN.

**Fig. 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 up-regulation of TH in PVN; 2) Decreased AMPK activation in VMH of Syn1-Cre;Ghsr<sup>f/f</sup> mice directly stimulates thermogenesis through sympathetic outflow; 3) Increased TH or decreased CB1 in PVN of Syn1-Cre;Ghsr<sup>f/f</sup> mice directly stimulates thermogenesis through sympathetic outflow; 4) Increased orexin in LH of Syn1-Cre;Ghsr<sup>f/f</sup> 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, SNS: Sympathetic Nervous System, NE: Norepinephrine.
Figure 1

(A) ATG STOP

(B) Relative GHS-R

(C) Food intake (g)

(D) Weight (g)

(E) Fat percentage (%)

(F) Glucose (mg/dl)

(G) Weight (g)

(H) Fat percentage (%)

(I) Glucose (mg/dl)

(J) Glucose (mg/dl)

(K) Glucose (mg/dl)

**Diabetes**
Figure 2

A) Mean RER

B) Food intake (g)

C) Food intake (g)

D) Food intake (g)
Figure 3

**A**
Food intake (kcal/day)

**B**
Heat (kcal/hr/kg BW)

**C**
Heat (kcal/hr/kg Lean)

**D**
Physical activity (count/hr)

**E**
RMR (kcal/hr/Lean)

**F**
Active ghrelin (pg/mL)

**G**
Wheel activity (counts)

**H**
Locomotor activity (count/hr)

**I**
Distance (m/day)

**J**
Locomotor activity (count/hr)

**K**
Temperature (°C)

**L**
Relative expression

**M**
Inguinal fat (BW %)

**N**
Active ghrelin (pg/mL)

**O**
Relative expression

**P**
Relative expression

**Q**
Relative expression
Figure 5

**Physical Activity**
- Energy Expenditure $\uparrow$
- Obesity & Insulin Resistance $\downarrow$

**Thermogenesis**
- DA signal $\uparrow$
- SNS $\uparrow$

**Energy Expenditure**
- GHS-R
- TH-DAT $\uparrow$

**Obesity & Insulin Resistance**
- LH
- CB1
- NPY/AgRP
- VMH
- AMPK
- ARC

**Diabetes**
- TH
- SNS
- Oxyxin $\uparrow$
- CB1
- VMH
- AMPK}$\downarrow$
- ARC
- NPY/AgRP
Supplementary figure legends

Supplementary Fig. 1
Neuronal Ghsr ablation does not affect the body length. (A), Body length, n=3-4. (B) Plasma IGF-1 in RD- and HFD-fed mice measured after overnight fasting. n=6-7.

Supplementary Fig. 2
The phenotype of Syn1-Cre;Ghsr^{f/f} mice fed regular diet (RD). Lean percentage (A), food intake (B), locomotor activity (C), energy expenditure (Heat) adjusted by body weight (BW) or lean mass (D), and resting metabolic rate (RMR) normalized by lean mass (E). n=6-7. *, p<0.05 **, p<0.001, Ghsr^{f/f} vs Syn1-Cre;Ghsr^{f/f}.

Supplementary Fig. 3
Neuronal Ghsr deletion does not affect insulin sensitivity under RD. ITT (A) and GTT (B) of mice fed RD for 22 weeks. ITT (C) and GTT (D) of mice fed RD for 44 weeks. n=5-7.

Supplementary Fig. 4
Neuronal Ghsr ablation increases voluntary physical activity in mice fed RD. Metabolic profile of RD-fed Syn1-Cre;Ghsr^{f/f} mice with running wheels. Five day recording of wheel rotations of Ghsr^{f/f} mice (A) and Syn1-Cre;Ghsr^{f/f} mice (B). Average daily running distance (C), locomotor activity (D), energy expenditure (Heat) adjusted by body weight (BW) or lean mass (E), and food intake during wheel running test (F). n=5-7, *, p<0.05, Ghsr^{f/f} vs. Syn1-Cre;Ghsr^{f/f}.

Supplementary Fig. 5
Neuronal Ghsr ablation improves thermogenesis in BAT and subcutaneous fat tissues under RD feeding. Rectal temperature change of RD-fed mice during 4°C cold challenge (A). Thermogenic gene expression in BAT (B) and subcutaneous fat (C), tissues were collected immediately after 4°C cold challenge. Beige markers in subcutaneous fat (D) and protein levels of UCP1 and UCP3 (E-G) in RD-fed mice. Beige markers in subcutaneous fat (H) and protein levels of UCP1 and UCP3 (I-K) in HFD-fed mice. n=5-7. *, p<0.05, **, p<0.001, Ghsr^{f/f} vs. Syn1-Cre;Ghsr^{f/f}.

Supplementary Fig. 6
Neuronal Ghsr ablation has no effect on energy expenditure under paired-feeding condition. The paired-feeding study was performed using 40 week-old RD-fed mice, n=4. Syn1-Cre;Ghsr^{f/f} mice were used as “Master”, having free access to food; Ghsr^{f/f} mice were used “Slave”, limiting their food intake to match that of the “Master” group.
Supplementary Figure 1

A

B

RD

HFD

Body length (mm)

IGF-1 (ng/ml)

Ghsr\textsuperscript{fl/fl}  
Syn1-Cre;Ghsr\textsuperscript{fl/fl}
Supplementary Figure 2

A

Lean percentage (%)

Ghsr\textsuperscript{f/f} Syn1-Cre;Ghsr\textsuperscript{f/f}

Age (weeks)

B

Food intake (Kcal/day)

\begin{align*}
\text{Ghsr}^{f/f} & \quad \text{Syn1-Cre;Ghsr}^{f/f} \\
\text{Light} & \quad \text{Dark} & \quad \text{Total} \\
\end{align*}

P=0.09

C

Locomotor activity (count/hr)

\begin{align*}
\text{Ghsr}^{f/f} & \quad \text{Syn1-Cre;Ghsr}^{f/f} \\
\text{Light} & \quad \text{Dark} & \quad \text{Total} & \quad \text{Light Fast} & \quad \text{Dark Fast} \\
\end{align*}

p=0.06

D

Heat (Kcal/hr/kg BW)

\begin{align*}
\text{Ghsr}^{f/f} & \quad \text{Syn1-Cre;Ghsr}^{f/f} \\
\text{Light} & \quad \text{Dark} & \quad \text{Light Fast} & \quad \text{Dark Fast} \\
\end{align*}

E

RMR (Kcal/hr/Lean)

\begin{align*}
\text{Ghsr}^{f/f} & \quad \text{Syn1-Cre;Ghsr}^{f/f} \\
\end{align*}

**
Supplementary Figure 3

A

B

C

D
Supplementary Figure 4

A) Running wheel (counts) for Ghsr<sup>fl/fl</sup>

B) Running wheel (counts) for Syn1-Cre;Ghsr<sup>fl/fl</sup>

C) Average distance (m/day) for Ghsr<sup>fl/fl</sup> and Syn1-Cre;Ghsr<sup>fl/fl</sup>

D) Locomotor activity (counts/hr) for Ghsr<sup>fl/fl</sup> and Syn1-Cre;Ghsr<sup>fl/fl</sup>

E) Heat (kcal/hr/kg BW) for Ghsr<sup>fl/fl</sup> and Syn1-Cre;Ghsr<sup>fl/fl</sup>

F) Food intake (Kcal/day) for Ghsr<sup>fl/fl</sup> and Syn1-Cre;Ghsr<sup>fl/fl</sup>
Supplementary Figure 5

A

Temperature (°C)

Hour

B

Relative expression

\[ \text{Ghsr}^{f/f} \]
\[ \text{Syn1-Cre}; \text{Ghsr}^{f/f} \]

C

Relative expression

\[ \text{Ghsr}^{f/f} \]
\[ \text{Syn1-Cre}; \text{Ghsr}^{f/f} \]

D

Relative gene expression

\[ \text{Ghsr}^{f/f} \]
\[ \text{Syn1-Cre}; \text{Ghsr}^{f/f} \]

E

Relative gene expression

\[ \text{Tmem26} \]
\[ \text{CD137} \]
\[ \text{Tbx1} \]

F

Relative gene expression

\[ \text{UCP1} \]
\[ \text{UCP3} \]
\[ \text{Actin} \]

G

Relative gene expression

\[ \text{RD} \]

H

Relative gene expression

\[ \text{Tmem26} \]
\[ \text{CD137} \]
\[ \text{Tbx1} \]

I

Relative gene expression

\[ \text{RD} \]

J

Relative gene expression

\[ \text{HFD} \]

K

Relative gene expression

\[ \text{HFD} \]
Supplementary Figure 6

- **Light**: Slave: Ghsr^{+/+}, Master: Syn1-Cre;Ghsr^{+/+}
- **Dark**: Slave: Ghsr^{+/+}, Master: Syn1-Cre;Ghsr^{+/+}

Heat (Kcal/hr/Kg Lean)
### Supplemental Table 1. Real-Time PCR Primer Sequences

| Gene   | Primer Sequences                        |
|--------|-----------------------------------------|
| 18S    | AGCCTGCGGCTTAATTTGAC                    |
|        | CAACTAAGAACGCGCAGCATGCA                 |
| PGC-1a | CATTGTATGCACTGAGCACGATGGA               |
|        | CGCTCAGGCAATGGAGAA                      |
| β3-AR  | TGCCCAACTCTGCTTCAACCCGCTC               |
|        | CGCTCACACTCATAGCAGCATCAAACC            |
| UCP1   | GTGAAAGTCAAGAATGCAAGC                   |
|        | AGGGCCCACTTCATAGGAGTC                   |
| UCP2   | TCACGTGCCCCTACCATCTG                   |
|        | AGGATGAAACCTTCTTCTGAGA                 |
| UCP3   | GAGCGGACCAGTCAGAGCCGTC                 |
|        | TGAGACTCAGAAGGCTTCCTC                   |
| NPY    | GCTAGGTAACAAACGAATGGGG                  |
|        | CACATGGAAGGCTTCTCAGA                   |
| AgRP   | GCAGACCCGAGCAGAAGAAGT                  |
|        | TGCGACTACAGGATGTTCCGTC                 |
| POMC   | GGGCCCTTCCCCCTAGAGTTCA                 |
|        | TTGATGATGCGCGTTCTGAA                   |
| CB1    | GCTGCAATCTGTGTGGCTCAG                   |
|        | TTGCCATCCTCTGAGGTGTG                   |
| AMPK1α | AAGGCAGAACGCAATGACATCA                 |
|        | CTTCCCTCTGACAGCCAAAT                   |
| mTmem26| ACCCTGTCATCCACAGAG                    |
|        | TGTTTGGTGGGATGTCCTAAGGTG               |
| Orexin | GCCGTCTCTACGAACTGTGTC                  |
|        | CGCTTTCCCAGATCAGGAGA                   |
| Leptin R| TGACCAGTGTAACAGTGCTAACTTCT             |
|        | CATATTAACGTAGGATGTCTGCTGACA            |
| STAT3  | CTTGCTCTCTCTACCCCAGACAT                |
|        | GATCCATGAAACGCTGGAGCG                  |
| DAT    | GGAAGCTGTCAGCCCGCTGAC                  |
|        | GAATGGCGCACTCCCCTCTG                   |
| TH     | GGTATACGCCAGCTGAAAG                    |
|        | TAGCCACAGTGACTCCAGA                    |
| D1R    | AACTGTATGTTGCTTCTGCTGTTG               |
|        | CATTCGTAGGTTGGTGCTGCCCG                |
| D2R    | CACTCGCCACTTCTGACATACA                 |
|        | TCTCCTCCGACACCTACCCCGA                 |
| BDNF   | GGGTCACAGCGGGAGATAA                    |
|        | GCCCTTGGGATACCGGGACTT                  |
| SF1    | TCCAGTACGGCAGGAAGAC                    |
|        | CTGTGCTCAGCTCCACCTC                   |
| IR     | CAAAGCACAACTGAGATGAGATGAC              |
|        | ACCACGTTGTCAGGTAATCC                   |
| mCD137 | CCAGTACCCATTCTGACTCCA                  |
|        | ATGAAGATCAGGCGAGAGCA                   |
| mTbx1  | TCGTGAGTGCT TTGCTCAGT                  |
|        | TGCCGTACGTCGCGAGGC                    |