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Ghrelin-responsive mediobasal hypothalamic neurons mediate exercise-associated food intake and exercise endurance

Omprakash Singh¹, Sean B. Ogden¹, Salil Varshney¹, Kripa Shankar¹, Deepali Gupta¹, Subhojit Paul¹, Sherri Osborne-Lawrence¹, Corine P. Richard¹, Nathan P. Metzger¹, Connor Lawrence¹, Luis Leon Mercado¹, Jeffrey M. Zigman¹,²,³,*

¹Center for Hypothalamic Research, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX
²Division of Endocrinology & Metabolism, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX
³Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX

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Address Correspondence To:
*Jeffrey M. Zigman, M.D., Ph.D.
University of Texas Southwestern Medical Center
5323 Harry Hines Blvd., Dallas, TX 75390-9077
USA
E-mail: jeffrey.zigman@utsouthwestern.edu
Abstract
Previous studies have implicated the orexigenic hormone ghrelin as a mediator of exercise endurance and the feeding response post-exercise. Specifically, plasma ghrelin levels nearly double in mice when they are submitted to an hour-long bout of high-intensity interval exercise (HIIE) using treadmills. Also, GHSR (ghrelin receptor)-null mice exhibit decreased food intake following HIIE and a diminished running distance (time until exhaustion) during a longer, step-wise exercise endurance protocol. To investigate whether ghrelin-responsive mediobasal hypothalamus (MBH) neurons mediate these effects, we stereotaxically delivered the inhibitory DREADD virus AAV2-hSyn-DIO-hM4(Gi)-mCherry to the MBH of Ghsr-IRES-Cre mice, which express Cre-recombinase directed by the Ghsr promoter. We found that chemogenetic inhibition of GHSR-expressing MBH neurons [upon delivery of clozapine-N-oxide (CNO)] 1) suppressed food intake following HIIE by 31.3%, 2) reduced maximum running distance by 20.7%-22.7% and raised blood glucose and blood lactate levels by 18.4%-51.5% and 24.6%-39.2%, respectively, during an exercise endurance protocol, 3) reduced food intake following ghrelin administration by 57.2%, but 4) did not affect glucose tolerance. Further, HIIE increased MBH Ghsr expression. These results indicate that activation of ghrelin-responsive MBH neurons is required for the normal feeding response to HIIE and the usual amount of running exhibited during an exercise endurance protocol.

Introduction
The mainly stomach-derived, acylated hormone ghrelin acts via growth hormone secretagogue receptors (GHSRs) to regulate food intake, blood glucose, and muscle function (1). Regarding food intake, ghrelin is orexigenic, potently stimulating eating when administered, ensuring appropriate rebound hyperphagia in response to short-term fasting, and engaging hedonic eating behaviors, for instance, in response to chronic psychosocial stress (2-4). Regarding blood glucose, administered ghrelin increases blood glucose while ghrelin deletion and GHSR deletion lead to progressive declines in blood glucose into the hypoglycemic range over the course of a week-long severe caloric restriction regimen (5-7). Regarding muscle function, administering ghrelin, GHSR agonists, and/or an agent that boosts plasma ghrelin ameliorate the reduced muscle contraction force and skeletal muscle wasting observed in rodent cachexia and muscle atrophy models and improve muscle strength in patients with cancer cachexia (8-13).

These actions of the ghrelin system are highlighted by exercise. For example, in a mouse chronic kidney disease model, ghrelin administration increases exercise endurance, gastrocnemius mass, and gastrocnemius muscle fiber size (13). Also, as we have reported, an hour-long bout of forced, high intensity interval exercise (HIIE) using treadmills nearly doubles plasma ghrelin levels in mice (14). This effect is transient, lasting at most 0.5-2 h (14). The effect of exercise on plasma ghrelin has also been investigated in humans using exercise regimens such as treadmills, cycling, and rowing; some studies report high plasma ghrelin and others report lower or unchanged ghrelin levels, as discussed previously (14). Also, whereas HIIE does not acutely increase food intake in wild-type mice over that observed in sedentary wild-type mice, GHSR-null mice exhibit marked reductions in food intake (by ~70%) in the first 4 h following HIIE as compared to exercised wild-type littermates (14). Further, although both GHSR-null mice and wild-type littermates are able to complete the HIIE protocol, when submitted to a longer, step-wise exercise endurance protocol, GHSR-null mice exhibit diminished endurance, reaching exhaustion after having run far less distance (by ~30%) and for far less time (by ~20%) than wild-type littermates (14). Moreover, plasma ghrelin levels measured at the time of exhaustion are positively correlated with distance run (14). These data suggest that exercise-induced increases in plasma ghrelin limit the capacity of exercise to restrict food intake following exercise, although they enhance exercise endurance.
Where ghrelin acts to have these effects on food intake following exercise and on exercise endurance is as yet unclear. However, several lines of evidence suggest that the mediobasal hypothalamus (MBH) is likely involved. For instance, both the VMH (ventromedial hypothalamus) and the ARC (arcuate hypothalamus), which comprise the MBH, regulate some metabolic responses to exercise (see discussion below) (15-19). Further, these MBH nuclei, as well as closely adjacent hypothalamic nuclei including the DMH (dorsomedial hypothalamus) and PMV (premammillary nucleus, ventral part), represent key sites of GHSR expression and ghrelin action (7, 20-25). In the present study, we aimed to determine whether GHSR-expressing neurons in the MBH mediate the effects of exercise on food intake and regulate exercise endurance.

Results
Confirmation of expected Cre recombinase activity within GHSR-expressing neurons of Ghsr-IRES-Cre mice
We began by confirming selectivity of Cre-recombinase activity to GHSR-expressing cells of a previously-reported Ghsr-IRES-Cre knock-in mouse line (21). This was achieved by crossing Ghsr-IRES-Cre mice to Cre-dependent ROSA26-YFP reporter mice and examining YFP expression within 10-12 week-old progeny containing one Ghsr-IRES-Cre allele and one ROSA26-YFP transgene. YFP expression within coronal brain sections extending approximately -1.34 mm to -3.08 mm from bregma (which spans the MBH to the midbrain) was compared to Ghsr mRNA expression as determined by in situ hybridization histochemistry (ISHH) using RNAscope in corresponding sections from NPY-hrGFP mice (26). Cre activity, as indicated by YFP reporter expression (Figure 1A-F), matched that of Ghsr mRNA expression (Figure 1G-L). As reported previously (20, 21, 27), regions containing GHSR-expressing cells included the ARC, VMH, DMH, PVH (paraventricular hypothalamus), LH (lateral hypothalamic area), PMV, PMD (premammillary nucleus, dorsal part), DTM (dorsal tuberomammillary nucleus), LM (lateral mammillary nucleus), MM (medial mammillary nucleus, medial part), ML (medial mammillary nucleus, lateral part), VTA (ventral tegmental area), and SN (substantia nigra). Isolated GHSR-expressing cells also were observed in the SuML (supramammillary nucleus, lateral part), SuMM (supramammillary nucleus, medial part), and EW (Edinger-Westphal nucleus).

Regarding the ARC, Ghsr mRNA expression was highly localized to NPY neurons, as indicated by co-localization with GFP (Figure 1M-O). Regarding the VMH, both Cre activity and Ghsr mRNA expression were observed sparsely in the dorsomedial (VMHdm) and central (VMHc) aspects, and more prominently in the ventrolateral (VMHvl) aspect and its capsule (VMHcap), as had been described earlier for Cre activity (21). Notably, ISHH studies using an 35S-labeled antisense GHSR riboprobe previously had demonstrated Ghsr mRNA expression in mouse VMH to be restricted to the VMHvl and VMHcap (20), although GHSR expression in the VMHdm and VMHc is well-established in the rat (20, 28). VMHvl expression of Ghsr mRNA within the mouse also has been established by RNA sequencing (29).

Additionally, although not done in our previous studies utilizing the Ghsr-IRES-Cre line (21, 30, 31), we performed dual-label histochemistry using MBH coronal sections to determine the overlap of Cre-dependent YFP-immunoreactivity in the Ghsr-IRES-Cre X ROSA26-YFP reporter mouse line with Ghsr mRNA expression, as determined using RNAscope in situ hybridization histochemistry. As indicated in Supplemental Figure 1A-D, 100% of YFP-expressing cells co-express Ghsr (as indicated by the overlapping red fluorescence RNAscope signal which represents antisense Ghsr riboprobes). Those same co-expression pattern is observed in a second representative mouse at two slightly more caudal levels (Supplemental Figure 1E-H, K-N). Notably, single-label YFP-immunohistochemistry and single-label Ghsr RNAscope in situ
hybridization histochemistry were also performed on two adjacent sections (see Supplemental Figures 1I and 1J, respectively), both of which were adjacent to the section that underwent dual-label histochemistry (Supplemental Figure 1E-H). A reduced number of YFP-immunoreactive cells is observed in the dual-labeled section than the section that underwent single-label YFP-immunohistochemistry, illustrating a known caveat of dual-label histochemistry to reduce the labeling of one or both signals.

Although 100% of YFP-expressing cells co-expressed the red fluorescence RNAscope signal, the RNAscope signal was also observed without co-localized YFP signal (Supplemental Figures 1D,H,N). In other words, Cre activity was localized only to Ghsr-expressing cells, but not all Ghsr-expressing cells expressed Cre activity. While it is not uncommon for Cre activity to be underrepresented in target cells (in this case, Ghsr-expressing cells), it is also important to mention two known caveats of the Ghsr-IRES-Cre mouse that might be impacting the histochemistry results. First, as originally reported (21), although we often observe a pattern of Cre activity in Ghsr-IRES-Cre mice that matches the known pattern of Ghsr mRNA expression, occasional Ghsr-IRES-Cre reporter mice have exhibited a somewhat asymmetric pattern (more expression on one side of the brain) or a less extensive bilateral pattern of Cre-activity (in which Cre-activity is missing from some of the usual sites of Ghsr expression). The reasons for these alternate patterns of Cre-activity in a subset of the reporter mice are unclear. Also, the original characterization of the Ghsr-IRES-Cre line demonstrated some slight differences in numbers of observed cells containing Cre-activity within certain brain regions, which seemed dependent on the reporter line used (21). Second, in a follow-up study, mice heterozygous for the Ghsr-IRES-Cre allele (as are the mice used here) exhibited a 34% reduction in the number of Ghsr mRNA-expressing cells within the ARC compared to wild-type littermates (31). That said, food intake and ARC c-fos induction in response to administered ghrelin were similar to that of wild-type mice, suggesting that the slight reduction in Ghsr expression in Ghsr-IRES-Cre mice did not impact responsiveness to ghrelin (31). Both of these caveats, together with reductions in observed signal that occur as a result of dual-label staining (see above), likely impact the observed degree of co-localization of Cre-driven YFP signal within cells containing Ghsr mRNA-associated red fluorescence.

**HIIE increases GHSR expression in the MBH**

To determine the effects of exercise on central GHSR expression, we submitted 10-12 week-old C57BL/6N to HIIE. Mice were familiarized to treadmills over 2 successive days and then were submitted to an HIIE protocol the next day. This consisted of withdrawing food 1 h after lights-on, and then 5 h later, submitting the mice to the 1 h HIIE bout, followed within 5 min by a tail nick to sample blood for glucose and lactate measurements and then immediate anesthetization and transcardial perfusion; sedentary control mice were treated similarly except they were kept sedentary during the period the others underwent HIIE (Figure 2A). As compared to sedentary control mice (Figure 2B-D; Supplemental Figure 2A-D), the exercised mice (Figure 2E-G; Supplemental Figure 2E-I) exhibited higher MBH expression of Ghsr mRNA, as determined using RNAscope in situ hybridization histochemistry. These changes were observed at all three levels of the MBH that were examined, including coronal sections located -1.34 mm, -1.82 mm, and -2.06 mm from bregma, within the ARC and VMH, but not within the DMH (Figure 2H-J). Quantification demonstrated that HIIE was associated with 79.4% and 132% increases in % fluorescent area (representing Ghsr expression) in the ARC and VMH, respectively.

Also, we confirmed the previous observation that HIIE acutely raises blood glucose (by 37.6% as compared to sedentary mice; Figure 2K) (14). Moreover, for the first time we show that HIIE also acutely raises blood lactate (by 104.3%; Figure 2L).
Inhibition of GHSR-expressing MBH neurons attenuates food intake after HIIE

We examined the functional significance of GHSR-expressing MBH neurons in mediating exercise-associated metabolic processes by first determining if inhibiting their activity, as achieved using a Cre-dependent chemogenetic system, reduces food intake after HIIE, similar to what is observed in GHSR-null mice (14). Stereotaxic surgery was used to deliver an inhibitory DREADD virus (AAV2-hSyn-DIO-hM4(Gi)-mCherry; hM4Di) bilaterally to the MBH of 9-11 week-old Ghsr-IRES-Cre, using coordinates that were chosen to target the ARC and adjacent nuclei, as had been achieved previously (21) (Figure 3A). Cre-expressing neurons infected with hM4Di express a designer receptor that engages downstream Gi-coupled signaling cascades, which in turn inhibit the activity of those neurons upon pharmacological engagement by clozapine-N-oxide (CNO); they also co-express an mCherry reporter that permits their identification (32, 33). Three weeks following stereotaxic injection of hM4Di, mice were familiarized to treadmills over 2 successive days and then were submitted to an HIIE protocol the next day. This consisted of withdrawing food 1 h after lights-on, delivering CNO (0.3 mg/kg BW i.p.) or saline 4 h later, submitting the mice to the 1 h HIIE bout 1 h after CNO or saline, followed within 5 min by a tail nick to sample blood for glucose and lactate measurements and re-introduction of standard chow diet to assess food intake over the next 4 h (Figure 3B). One week later, this protocol was repeated on the same mice using a crossover design to deliver saline or CNO.

Following subsequent exercise endurance and administered ghrelin-induced food intake and c-fos studies (see below), mCherry expression was determined to classify those cases with correctly-targeted virus injections as “hits” or mis-targeted virus injections as “misses.” “Hits” were defined here as cases with mCherry expression either bilaterally or unilaterally in both the ARC and VMH but without mCherry expression caudal to the ARC (e.g. caudal to a distance -2.46 mm from bregma); “hits” most often also expressed mCherry in the DMH, MTu, PMV, +/- the PH. “Misses” were defined here as cases without mCherry expression in both the ARC and VMH; “misses” most often expressed mCherry caudal to a distance -2.46 mm from bregma, often also expressed mCherry in the PMV, and occasionally included cases with minimal mCherry expression in either the ARC or VMH (but not both). mCherry expression within three levels of the MBH of a representative “hit” is depicted in Figure 3C-E, whereas the lack of mCherry expression in the corresponding MBH levels of a representative “miss” is depicted in Figure 3F-H. A more complete survey of mCherry expression within 5 different coronal brain section levels (extending from a distance of -1.34 mm to -2.80 mm from bregma) is included in Supplemental Figure 3 for all 16 “hits”, in Supplemental Figure 4 for all 11 “misses”, and in Table 1. Of those 16 “hits”, 13 showed bilateral targeting whereas 3 (Supplemental Figure 3M, O, and P) showed unilateral targeting.

Food intake over the first 4 h after HIIE was significantly attenuated by CNO treatment when compared to saline treatment in the “hits”, with the difference between CNO and saline treatment becoming most prominent at 4 h (31.3% reduction; Figure 3I). Notably, separate analysis of only those 13 (out of 16) “hits” targeted bilaterally showed similar results; namely, CNO treatment reduced food intake after HIIE by 31.8% (P<0.01) as compared to saline (data not shown). CNO treatment did not reduce or otherwise affect exercise-induced food intake in the “misses” (Figure 3M). Neither blood glucose, blood lactate, nor body weight was affected by CNO delivery, in either “hits” or “misses” (Figure 3J-L, N-P).

Inhibition of GHSR-expressing MBH neurons impairs exercise endurance and MBH c-fos induction resulting from exercise

Next, we determined if chemogenetic inhibition of GHSR-expressing MBH neurons reduces exercise endurance, similar to what is observed in GHSR-null mice (14). One week following the
above-described set of HIIE studies in hM4Di-injected mice (Figure 3), access to food was restricted beginning 4 h after lights-on, CNO (0.3 mg/kg BW i.p.) or saline was delivered 1 h later, mice were submitted to a step-wise exercise endurance protocol (lasting ~130 min, at most) 1 h following CNO or saline, after which tails were nicked to obtain blood for glucose, lactate, and ghrelin measurements (Figure 4A). One week later, this protocol was repeated on the same mice using a crossover design to deliver saline or CNO.

Exercise endurance was significantly attenuated by CNO treatment when compared to saline treatment in the “hits”, as evidenced by the CNO-treated “hits” reaching exhaustion after having run only 79.3% as far (Figure 4B) and 85.3% as long (Figure 4C) as saline-treated “hits”. Notably, separate analysis of only those 13 (out of 16) “hits” targeted bilaterally showed similar results; namely, CNO treated bilateral “hits” reached exhaustion after having run only 77.2% as far (P<0.0001; data not shown). Further, CNO-treated “hits” achieved maximal running speeds that were only 83.8% as fast (Figure 4D) as saline-treated “hits”. Additionally, blood glucose was higher by 18.4% (Figure 4E) and blood lactate was higher by 24.6% (Figure 4F) at exhaustion as a result of CNO treatment in “hits”. In the “misses”, CNO treatment did not affect maximal running distance, total running duration, maximal running speed, or blood glucose at exhaustion (Figure 4H-L), although it did reduce blood lactate at exhaustion (by 22.1%; Figure 4L). Although we do not have an explanation for the CNO-associated lactate reduction in the “misses”, it did not impact their exercise endurance. CNO treatment did not impact plasma ghrelin at exhaustion in either “hits” or “misses” (Figure 4G, M).

A separate set of exercise endurance studies was performed using a different set of controls. Whereas the above mice all were injected with hM4Di and then assessed in a crossover fashion following CNO vs. saline, here, mice were injected with one of two viruses (hM4Di or a non-inhibitory control virus) and then all were assessed after CNO administration. Specifically, CNO (0.3 mg/kg BW i.p.) was administered to 12-14 week-old Ghsr-IRES-Cre mice which had received bilateral MBH stereotaxic injections of either hM4Di or a control AAV-hSyn-DIO-mCherry (“Cre-dependent mCherry control”) virus four weeks earlier (Figure 5A). Afterwards, the same protocol as described above (Figure 4A) was used except a) 1 wk earlier, the mice were submitted to an oGTT protocol (see below), b) each of the two exercise endurance runs was proceeded by CNO (instead of once with CNO and once with saline), c) plasma LEAP2 at exhaustion also was checked, and d) the mice were anesthetized and transcardially perfused with formalin immediately following the blood collection (Figure 5B). Post-hoc immunohistochemical analysis of coronal brain sections identified four Cre-dependent mCherry control “hits” and five hM4Di “hits” (Figure 5C-H; Supplemental Figure 5).

Just as had been observed in hM4Di-injected “hits” after CNO treatment vs. saline (Figure 4), CNO treatment reduced maximal running distance (by 22.7%; Figure 5I), total running duration (by 14.5%; Figure 5J), and maximal running speed (by 14.5; Figure 5K) in hM4Di-injected “hits” vs. Cre-dependent mCherry control virus-injected “hits”. Also similar to hM4Di-injected “hits” after CNO treatment vs. saline (Figure 4), CNO treatment increased blood glucose (by 51.5%; Figure 5L) and blood lactate (by 39.2%; Figure 5M) at exhaustion in hM4Di-injected “hits” vs. Cre-dependent mCherry control virus-injected “hits”. Neither plasma ghrelin at exhaustion, plasma LEAP2 at exhaustion, nor the LEAP2:ghrelin molar ratio (which helps determine the degree of ghrelin resistance) (34) at exhaustion was impacted by chemogenetic inhibition of GHSR-expressing MBH neurons (Supplemental Figure 6A-C). However, when we grouped the data from all hM4Di-injected “hits” after CNO treatment from Figures 4-5 and Supplemental Figure 6, we demonstrated that plasma ghrelin positively correlated with distance run (just as had been shown previously in wild-type mice (14), blood glucose negatively correlated with
distance run, and blood lactate did not correlate with distance run (Figure 5N-O; Supplemental Figure 6D).

Further, CNO treatment of hM4Di-injected “hits” reduced the amount of neuronal activation in the MBH observed at exhaustion as compared to CNO treatment of Cre-dependent mCherry control virus-injected “hits”. Specifically, the numbers of c-fos-immunoreactive cells in the ARC and VMH were reduced by 20.3 % and 22.4%, respectively, in the hM4Di-injected “hits” vs. the control virus-injected “hits” (Figure 5P-Q). The percentage of c-fos-immunoreactive cells in the VMH that co-expressed mCherry was reduced by 88.9% in the hM4Di-injected “hits” vs. the control virus-injected “hits” (Figure 5R). No differences were observed in c-fos-immunoreactivity within the DMH or PMV (Supplemental Figure 6E-F).

To better characterize the chemical phenotypes of the GHSR-expressing neurons of the hM4Di-injected “hits”, we assessed their expression of nNOS (neuronal nitric oxide synthase), which, within the MBH, is most highly expressed in the VMHvl (35). Approximately 63.3% of VMHvl cells expressing mCherry (in other words, 63.3% of the GHSR-expressing VMHvl neurons inhibited upon CNO administration) co-expressed nNOS-immunoreactivity; about 26% of nNOS-immunoreactive cells in the VMHvl co-expressed mCherry (Figure 6).

**Inhibition of GHSR-expressing MBH neurons reduces food intake and MBH c-fos induction in response to administered ghrelin**

To demonstrate that targeting the same GHSR-expressing neurons affecting HIIE-induced food intake and exercise endurance are actually ghrelin-responsive, we determined whether their chemogenetic inhibition would also attenuate food intake and ARC c-fos induction in response to ghrelin administration. Thus, one week following the above-described set of exercise endurance studies in hM4Di-injected mice (Figure 4), access to food was restricted beginning 4 h after lights-on, CNO (0.3 mg/kg BW i.p.) was delivered 1 h later, ghrelin (1 mg/kg BW s.c.) was administered 1 h later, and food intake was measured for 2 h, after which mice were deeply anesthetized and transectively perfused in preparation for assessment of c-fos induction (Figure 7A). Just as had previously been observed (21), ghrelin-induced food intake was significantly attenuated (by 57.2%) in “hits” as compared to “misses” following CNO treatment (Figure 7B). This coincided with a 71.4% reduction (Figure 7C) in the number of c-fos-immunoreactive cells within the ARC of “hits” (Figure 7D-E) vs. “misses” (Figure 7F-G).

**Inhibition of GHSR-expressing MBH neurons does not affect glucose tolerance**

Given the increased blood glucose observed in exercised mice with inhibited GHSR-expressing MBH neurons (Figure 4E and 5L), we next determined if inhibition of GHSR-expressing MBH neurons would impact oral glucose tolerance in the sedentary state. These studies were also supported by previous work demonstrating that ghrelin deletion and GHSR deletion lower fasting blood glucose, improve glucose tolerance, enhance insulin sensitivity, and/or increase glucose-stimulated insulin secretion (36-38). Further, chemogenetic inhibition of steroidogenic factor-1 (SF1)-expressing VMH neurons previously had been shown to worsen glucose tolerance (39). For these studies, we used a new set of 12-14 wk-old Ghsl-IRES-Cre mice which had received bilateral MBH stereotactic injections of a control AAV-hSyn-mCherry (“non-Cre-dependent mCherry control”) virus (Figure 8A) three weeks earlier (n=4 “hits”, Figure 8C) and the same cohort of “Cre-dependent mCherry control virus”-injected (Figure 8D) vs. hM4Di-injected Ghsl-IRES-Cre mice (Figure 8E) used in Figure 5. As depicted in Figure 8B, food access was restricted 3 h following lights-on, CNO (0.3 mg/kg BW i.p.) was administered 5 h afterwards, and then 1 h later, 2 mg/kg BW glucose was administered by oral gavage. Blood glucose was assessed 5 min prior to and again at 15, 30, 60, 90, and 120 min following glucose delivery. Blood glucose curves are indicated in Figure 8F. There were no differences among the
groups in terms of blood glucose levels obtained just prior to glucose administration (after a 6 h fast) (Figure 8G) or area under the curve (Figure 8H).

Discussion
These studies reveal that HIIE increases Ghsr expression in the MBH (by 79.4% in the ARC and by 132% in the VMH), similar to the HIIE-associated increase in plasma ghrelin reported previously (14). These studies also reveal that activation of ghrelin-responsive, GHSR-expressing MBH neurons is required for the normal feeding response to HIIE and the usual amount of endurance and the usual blood glucose and lactate responses exhibited by mice during a step-wise exercise endurance protocol. Specifically, DREADD-assisted inhibition of GHSR-expressing MBH neuronal activity suppressed food intake following HIIE (by 31.3%) and maximal running distance (by 20.7%-22.7%), total running duration (by 14.5%-14.7%), and maximum running speed (by 14.5%-16.1%) during the exercise endurance protocol (hm4Di-injected “Hits” treated with CNO vs. saline; hm4Di-injected, CNO-treated “hits” vs. Cre-dependent mCherry control virus-injected, CNO-treated “hits”). The reduction in exercise endurance coincided with a 22.4% and 20.3% reduction in the numbers of c-fos-immunoreactive cells in the VMH and ARC, respectively (hm4Di-injected “hits” vs. Cre-dependent mCherry control virus-injected “hits”). Also, DREADD-assisted inhibition of GHSR-expressing MBH neuronal activity increased blood glucose (by 18.4%-51.5%) and blood lactate (by 24.6%-39.2%) following the exercise endurance protocol (hm4Di-injected “hits” treated with CNO vs. saline; hm4Di-injected, CNO-treated “hits” vs. Cre-dependent mCherry control virus-injected, CNO-treated “hits”). Those “hits” with higher plasma ghrelin at exhaustion exhibited greater exercise endurance whereas those “hits” with higher blood glucose exhibited lower exercise endurance. hm4Di-injected “hits” treated with CNO also exhibited a 57.2% reduced food intake and a 71.4% reduced c-fos induction within the ARC in response to ghrelin administration as compared to “misses” treated with CNO, confirming that the neuronal population being interrogated is responsive to ghrelin and confirming previous studies (21). Chemogenetic inhibition of GHSR-expressing MBH neuronal activity did not affect glucose tolerance. Further, ~63.3% of hm4Di-infected, GHSR-expressing VMHvl neurons co-expressed nNOS-immunoreactivity, suggesting a possible role for this nNOS subpopulation in mediating HIIE-induced food intake, exercise endurance, and administered ghrelin-induced food intake.

As hinted in the Introduction, evidence in the literature had supported the premise suggesting a role of MBH neurons — including both those in the VMH and the ARC — in ghrelin’s exercise-related effects. VMH-specific deletion of the transcription factor steroidogenic factor 1 (SF1) reduces exercise endurance and impairs exercise-associated mobilization of several species of free fatty acids (16). Deletion of VMH SF1, which usually is induced by prolonged exercise training, blunts fat mass reductions, blood glucose improvements, and energy expenditure increases associated with exercise training (16). VMH SF1 deletion attenuates the usual metabolic responses of skeletal muscle to exercise, including increases in the mass of several skeletal muscles and induction of muscle PGC-1α expression (16). Further, VMH ablation and blockade of VMH neuronal activity, which may inhibit sympathetic outflow, reduce exercise-induced increases in circulating free fatty acids and their utilization (17, 18). Regarding a role of the ARC, exercise induces plasticity within ARC neuronal circuits (19). Specifically, whole-cell patch-clamp recordings indicate that ARC AgRP neurons from exercised mice exhibit a hyperpolarized resting membrane potential, less frequent spontaneous excitatory synaptic currents (sEPSCs), more frequent spontaneous inhibitory synaptic currents (sIPSCs), and decreased action potential frequency (19). In contrast, ARC POMC neurons from exercised mice exhibit a depolarized resting membrane potential, more frequent sEPSCs, and increased action potential frequency (19). Further, in vivo fiber photometry indicates that HIIE decreases whole cell calcium levels in ARC AgRP neurons and increases those levels in POMC neurons.
These data support a model by which exercise leads to a rapid reorganization of synaptic inputs and biophysical properties of ARC neurons. It is notable that ghrelin, the circulating levels of which rise as a result of HIIE and correlate with distance run in the exercise endurance test, also increases the frequency of sIPSCs onto ARC POMC neurons and excitatory currents in AgRP neurons (40, 41).

Prior evidence for direct engagement of MBH neurons by ghrelin is extensive, especially those populating the ARC (1, 42). As just a few examples, not only are GHSRs highly expressed within the MBH, as demonstrated previously and confirmed here, but also administration of ghrelin and GHSR agonists markedly induce c-fos within ARC AgRP neurons (22, 43-45). Selective GHSR expression in ARC AgRP neurons is sufficient to allow ghrelin to induce food intake and normalizes the relative hypoglycemia observed in fasted GHSR-null mice (7). Conversely, AgRP neuron-selective GHSR deletion as well as ablation of ARC AgRP neurons abolish ghrelin’s acute orexigenic effects (24, 46). GHSR expression also occurs in the VMH, including SF1-neurons (21, 47). In rats, in which VMH GHSR expression is much more prominent than in mice (see Results section for further discussion), VMH inhibition of AMPK robustly impairs the central orexigenic effect of ghrelin (23). Further, antisense GHSR shRNA-mediated knockdown of GHSR expression in the VMH reduces wheel running activity in both ad libitum-fed rats and rats subjected to a restricted feeding schedule, while also attenuating body weight loss otherwise induced by the wheel running activity (48). Rats with GHSR knockdown in the VMH also exhibit a delay in the onset of the food anticipatory activity that characteristically occurs prior to food availability under the restricted feeding schedule (48). Interestingly, VMH GHSR knockdown in ad libitum-fed rats increases food intake and body weight gain (48).

Thus, we are reassured by the findings here demonstrating effects of chemogenetic inhibition of GHSR-expressing MBH neurons to reduce eating after HIIE and after administered ghrelin and to reduce exercise endurance. As no cases had hM4Di targeted selectively to just one of the regions comprising the MBH, further work is needed to distinguish the roles of GHSR-expressing neurons in the ARC from those in the VMH or from those in one of the other MBH-adjacent sites with GHSR-expressing neurons. Additional studies also are needed to confirm if the effects of chemogenetic inhibition are the result of blocking ghrelin action on those neurons as opposed to a more generalized effect on the activity of those neurons unrelated to ghrelin or GHSR constitutive activity (1). Certainly, the fact that chemogenetic inhibition of the GHSR-expressing MBH neurons reproduces the exercise phenotype of GHSR-null mice suggests that ghrelin and GHSR indeed are involved. Yet, studies using Cre-mediated GHSR deletion within the MBH or within a specific MBH region or specific MBH neuronal subtype, would undoubtedly help facilitate confirmation of that hypothesis.

Although the current study establishes key effects of GHSR-expressing MBH neurons to impact metabolic changes and exercise endurance, it only scratches the surface regarding the downstream mechanisms by which these occur. The observed changes in blood lactate and blood glucose levels are among the clues. In exercise-endurance tested mice, blood lactate levels were higher at the point of exhaustion when GHSR-expressing MBH neurons were chemogenetically inhibited. This finding suggests that activation of these neurons directs lactate utilization, facilitating greater endurance; in contrast, inhibition of these neurons would lead to under-utilization of lactate, thereby reducing endurance. Indeed, improved metabolism of lactate is just one of many adaptations that enable endurance athletes to sustain work (49). Blood glucose levels were also higher at the point of exhaustion when GHSR-expressing MBH neurons were chemogenetically inhibited, and there was a negative correlation between blood glucose and distance run in the exercise endurance protocol. These blood glucose data suggest that the GHSR-expressing MBH neurons direct glucose utilization as a fuel source to help mice
run farther. In mice with inhibited GHSR-expressing MBH neurons, glucose is not efficiently utilized, leading to higher blood glucose levels and potentially decreased endurance. Further research, including more in-depth examination of lactate and glucose kinetics following exercise, is needed to test these hypotheses. It also would be worthwhile for future studies to explore why blood lactate was lowered by CNO administration in the “misses”, in which the activity of GHSR-expressing neurons outside the MBH was impacted but not their exercise endurance.

Another clue regarding potential downstream mediators comes from the novel observation that ~63.3% of hM4Di-infected, GHSR-expressing VMHvl neurons contain nNOS. This subpopulation of GHSR-expressing neurons is part of the total population of GHSR-expressing MBH neurons which, when chemogenetically inhibited, reduced food intake after HIIE, exercise endurance, and administered ghrelin-induced food intake. Important metabolic and behavioral effects of nNOS in the VMH have previously been demonstrated. For instance, nNOS-derived nitric oxide (NO) production in the VMH is stimulated by insulin-induced hypoglycemia, is required for glucose sensing by VMH glucose-inhibited neurons, and is necessary for the usual counter-regulatory response to hypoglycemia (50, 51). Most nNOS-expressing VMHvl neurons have been characterized as glutamatergic and ERα-positive (35), and ERα-positive VMHvl neurons play key roles in sensing glucose fluctuations and preventing severe hypoglycemia (52). Further, chemogenetic activation of ERα + melanocortin 4 receptor (MC4R) co-expressing VMHvl neurons markedly increases spontaneous locomotor activity in male mice, female mice with intact ovaries, and estrogen-depleted ovariectomized female mice, which otherwise are less active than intact females (29). It is as yet unclear if the GHSR + nNOS co-expressing VMHvl neurons observed here overlap with these previously-described ERα + MC4R co-expressing VMHvl neurons. Future studies are needed to specifically investigate the role of nNOS and ERα in ghrelin’s effects on food intake after HIIE and exercise endurance.

We would be remiss in not mentioning some of the caveats of the exercise protocols used here. Although both the HIIE and exercise endurance protocols were preceded by two adaptation days in which the mice were familiarized to the treadmills (which included 5 min at rest on the treadmills and then exercising them for 5 min x 8-10 m/min and then for 5 min x 10-12 m/min), the mice did not otherwise undergo a preceding training period, unlike most human athletes. Also, the mice were coaxed to continue running on the treadmill with the assistance of an electric stimulus generated by a shock grid present at the treadmill base (HIIE and exercise endurance) and by manually tapping their tails using a soft nylon bottle brush (HIIE), which could be viewed as stress-, pain-, and/or fear-inducing. These prompts likely overlap only in part with the various motivational aides at play in human athletes. Additionally, the exercise protocols reported here were performed during the daytime, when mice usually have low levels – although not zero levels – of spontaneous physical activity. Despite these caveats, it is reasonable to assume that the same neurocircuits likely would be engaged had the exercise protocols been possible to perform without a shock grid or bottle brush and had they been performed during the nighttime using trained mice. Future studies that incorporate those additional elements into the HIIE and exercise endurance protocols could test such a hypothesis.

**Material and Methods**

**Mice**

All experiments were approved by the Institutional Animal Care and Use Committee of UT Southwestern (UTSW) Medical Center. *Ghsr-IRES-Cre* mice (containing one copy of the *Ghsr-IRES-Cre* allele) (21), mice derived from crosses between *Ghsr-IRES-Cre* mice and ROSA26-YFP mice [B6.129X1-Gt(Rosa)26So<sup>tm1(EYFP)Cos</sup>/J] (The Jackson Laboratory; stock number
006148) containing one copy of the Ghsl-IRESCre allele and one ROSA26-YFP transgene, NPY-hrGFP (26), and C57BL/6N mice were used in this study. All lines had been backcrossed > 10 generations onto a C57BL/6N genetic background. All studies were performed using male mice housed at standard room temperature (22-24°C) under a 12 h dark-light cycle with ad libitum access to water and standard chow diet [2916 Teklad Global 16% protein diet (Envigo, Indianapolis, IN)], except as indicated. Diagrammatic representations of the experimental protocols were prepared using CorelDraw 11 software (Corel Inc., Austin, TX).

Stereotaxic surgeries and viral injections
Stereotaxic surgery was performed as previously described (21), with some modifications, on mice under ketamine (120 mg/kg BW)/xylazine (16 mg/kg BW) i.p. anesthesia or 1.5% isoflurane gas anesthesia while restrained in a Kopf stereotaxic apparatus. Following standard disinfection procedures, a small incision (∼1.0 cm) was made into the skin overlying the skull, a small hole was drilled into the skull using a high-speed rotary micromotor (Foredom, Bethel, CT), and 200 nL of virus was injected into one side of the brain (see coordinates below) over a period of 10 min using a pulled glass micropipette connected to an air pressure injector system. A micromanipulator (Model S48 Stimulator, Grass Technologies, West Warwick, RI) was used to control injection speed at 20 nL min⁻¹. After a 10 min waiting period, the micropipette was slowly retracted and the procedure was repeated on the contralateral side. The incision site was closed using a surgical suture. The mice were monitored on a warming pad until awake, after which they were singly-housed and administered buprenorphine 1 mg/kg BW s.c. every 12 h for 24 h and carprofen 5 mg/kg BW s.c. daily for 3 days to relieve pain. Mice were allowed to recover for 3 wks prior to undergoing behavioral/physiological testing. Cre-dependent AAV2-hSyn-DIO-hM4D(Gi)-mCherry virus (32) (hereafter referred to as hM4Di; Cat# 44362; Addgene, Watertown, MA; titer: 2.3E13 GC/mL) was targeted bilaterally to the MBH by use of the following brain coordinates based on a mouse brain atlas (53): (distance from bregma: –1.40 mm; lateral from midline: +/–0.20 mm; ventral from brain surface: –5.65 mm). These mice were used to generate the data in Figures. 3, 4, and 7. In a separate cohort of mice, AAV2-hSyn-mCherry virus (“non-Cre-dependent mCherry control virus”; Cat# 114472; Addgene; titer: 1.8E13 GC/mL), AAV2-hSyn-DIO-mCherry (“Cre-dependent mCherry control virus”; Cat# 50469; Addgene; titer: 2.1E13 GC/mL), or hM4Di were targeted slightly less ventrally to the following brain coordinates: (distance from bregma: –1.40 mm; lateral from midline: +/–0.20 mm; ventral from brain surface: –5.60 mm). These mice were used to generate the data in Figures 5, 6, and 8. Illustrations of the stereotaxic injections into the MBH were made using CorelDraw 11 software, and are inspired by the mouse brain atlas of Paxinos and Franklin (53).

High intensity interval exercise (HIIE) protocol
The high intensity interval exercise (HIIE) protocol involved running mice on motorized treadmills (Exer-6; Columbus Instruments, Columbus, OH) as previously described(14, 54) (Figure 3B). Mice were first familiarized to the treadmills for 2 days prior to the exercise bout [Day 1: 5 min rest on the treadmill followed by running for 5 min at the speed of 8 m/min and then for 5 min at the speed of 10 m/min; Day 2: 5 min rest on the treadmill followed by running for 5 min at the speed of 10 m/min and then for 5 min at the speed of 12 m/min]. On Day 3, mice were subjected to an HIIE bout, as follows. Food was removed from home cages at the start of the light cycle (7 AM) for a duration of 6 h. At the 4th h of food restriction, mice were administered saline or CNO (0.3 mg/kg BW i.p.; Cat# C0832; Sigma-Aldrich, St. Louis, MO) in a crossover fashion. One hour following saline or CNO administration, mice were rested on the treadmill for 5 min. Immediately after the 5 min of rest, they were submitted to a 1 h exercise bout consisting of 3 x 20-minute intervals (5 min at a speed of 12 m/min, followed by 10 min at a speed of 17 m/min, and then 5 min at a speed of 22 m/min), without rest between intervals. Mice were coaxed to continue running on the treadmill by means of an electric stimulus (0.25
mA X 163 V and 1Hz) generated by a shock grid present at the treadmill base and by manually tapping their tails using a soft nylon bottle brush, as needed. Notably, during the HIIE protocol, all mice received one (not more and not less) electric shock during each of the three 20 min intervals when running at 22 m/min speed; rarely did we witness mice receiving an electric shock at the lower speeds. After completion of the HIIE bout, blood glucose and blood lactate concentrations were determined immediately from blood taken from tail snips using a Bayer Contour glucometer (Parsippany, NJ) and a Nova Biomedical Lactate Plus™ meter (Mörfelden- alldorf, Germany), respectively, mice and standard chow (Teklad Global Diet [2916]) were re-introduced into the home cages, and food intake was measured over the next 30 min, 1 h, 2 h and 4 h.

Exercise endurance protocol
Exercise endurance was tested by subjecting mice to a stepwise running paradigm as described (14) with minor modifications (Figure 4A). The mice were first acclimatized to the treadmill for 2 days, as described above for the HIIE protocol. On the day of the experiment (Day 3), food was removed from home cages 4h after the start of the light cycle (7 AM) for a duration of 2 h. Five hours after the start of the light cycle (12 PM), mice were injected with either saline or CNO (0.3 mg/kg BW i.p.). One hour after the injections, mice were placed on the treadmill for 5 min at rest, followed by running with a starting speed of 10 m/min for 40 min, next by running at speeds that were increased at the rate of 1 m/min every 10 min until the speed reached 13 m/min, and finally by running at speeds that were increased at the rate of 1 m/min every 5 min until exhaustion. The exhaustion time was noted as the time at which the mice stopped running and remained on the electric shock grid for more than 5 s, without attempting to resume running (14, 19). Bottle brushes were not used to coax the mice to run. Just after exhaustion, mice were removed from the treadmill and blood was collected from tail snips to measure blood glucose, blood lactate, and ghrelin.

Notably, although we did not measure hormone levels following the exercise endurance protocol in the current study, in our prior study using this same exercise endurance protocol (14), marked, genotype-independent rises in plasma corticosterone were noted at the point of exhaustion in both wild-type mice and GHSR-null littermates, the latter of which exhibited decreased running distance. Further, there was an effect of exercise to raise plasma norepinephrine but not plasma epinephrine when measured at the point of exhaustion, although exhausted GHSR-null mice had significantly lower epinephrine and norepinephrine levels than exhausted wild-type mice (14). Also, when wild-type mice were time-matched to run only as long as GHSR-null mice (to the point of exhaustion of the GHSR-null mice), epinephrine and norepinephrine levels were found to be significantly lower in the exhausted GHSR-null mice (14).

Ghrelin induced food intake studies
Ghrelin-induced food intake was performed as in (21) (Figure 7A) and as described in detail in the Supplemental Methods.

Oral glucose tolerance test (oGTT)
oGTT was performed as in (39) (Figure 8B) and as described in detail in the Supplemental Methods.

Determination of plasma ghrelin and LEAP2
Tail vein blood was collected and processed as described in the Supplemental Methods.

RNAScope in situ hybridization histochemistry for Ghsr
NPY-GFP (Figure 1G-O), C57BL/6N (Figure 2B-G; Supplemental Figure 2), and mice carrying both a Ghsr-IRES-Cre allele and a Rosa26-YFP transgene (Figure 1A-F; Supplemental Figure 1A-N) were deeply anesthetized with chloral hydrate (500 mg/kg BW i.p.) and perfused transcardially with diethyl pyrocarbonate (DEPC)-treated 0.9% phosphate-buffered saline (PBS) followed by 10% neutral buffered formalin, using high precision multichannel pump (Ismatec; USA)(45, 55). Brains were removed and post fixed in 10% formalin for overnight at 4°C and then cryoprotected in 25% sucrose solution in DEPC-treated PBS overnight at 4°C. After embedding in Tissue-Tek OCT compound, serial 25-µm thick coronal sections extending from the olfactory bulb to the cervical spinal cord were obtained using a cryostat (Leica), immersed in DEPC-treated PBS buffer, and separated into five equal brain series. One series of hypothalamic sections containing the ARC, extending from -1.34 mm to -3.08 mm past bregma, was rinsed in DEPC-PBS, treated with 0.9% hydrogen peroxide for 10 minutes at room temperature, rinsed, mounted onto SuperFrost slides (Fisher Scientific, Pittsburgh, PA), and then dried overnight in a vacuum oven at 37°C. The next day, RNAScope in situ hybridization histochemistry was performed using the RNAscope® Multiplex Fluorescent Kit v2 assay (Advanced Cell Diagnostics), as per the manufacturer’s instructions: Slides were rinsed 2 times in PBS and placed in an oven for 30 min at 60°C. Afterwards, slides were post fixed in 10% formalin for 15 minutes at 4°C then gradually dehydrated in ethanol (50, 70 and 100%, 5 min each) before target retrieval for 15 minutes at 98-102°C. Slides were incubated in protease III (#322337, ACD) for 30 min at 40°C then washed in distilled water and incubated in RNAScope probes for Ghsr (Mm-GHSR; #426141, ACD) for 2 h at 40°C. Sections were further processed using the RNAscope® Multiplex Fluorescent Detection Reagents v2 kit (#323110, ACD) using instructions provided by the manufacturer. Slides were immediately coverslipped using EcoMount medium (Biocare; EM897L).

**Tissue processing for immunofluorescence**

Mice were deeply anesthetized with chloral hydrate (500 mg/kg BW i.p.) and transcardially perfused with 0.9% PBS followed by 10% neutral buffered formalin, using a high precision multichannel pump, as described previously (45, 55) with some modifications. Brains were dissected, postfixed in the same fixative overnight at 4°C, and then cryoprotected by immersing in 25% sucrose solution in PBS overnight at 4°C. After embedding in Tissue-Tek OCT compound, serial 25-µm thick coronal sections extending from the olfactory bulb to the cervical spinal cord were obtained using a cryostat, immersed in antifreeze solution, separated into five equal brain series, and then stored at -20°C until further processing. One series of hypothalamic sections containing the ARC, extending from -1.34 mm to -2.80 mm past bregma, was washed with PBS and then mounted on SuperFrost slides, dried overnight and coverslipped with Vectashield mounting media with 4',6-diamino-2-phenylindole (DAPI) [Cat # H-1200, Vector Labs, Burlingame, CA]. mCherry fluorescence was determined at the end of the study to classify those cases with appropriately targeted virus injections as “hits” or mis-targeted virus injections as “misses”, as defined in the Results section.

Separate series of free-floating coronal sections -- one series containing the sections approximately -1.82, -2.06 mm (Figure 5C-D, F-G and Figure 7D-G) and -2.30 mm (Figure 5E, H) past bregma and another series containing a section approximately -1.94 mm past bregma (Figure 6) -- were processed for c-fos and nNOS, respectively, as previously described (45), with minor modifications. Sections were rinsed in PBS, immersed in 0.5% Triton X-100 solution in PBS for 30 min, and blocked in 3% normal donkey serum (Cat # 017-000-121, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS for 2 h. Sections were incubated either in diluted rabbit anti-c-fos antibody (Cat # ab190289; Abcam, Waltham, MA; dilution 1:1000) or diluted rabbit anti-nNOS antibody (Cat # 61-7000; Invitrogen, Carlsbad, CA; dilution 1:1000) for 24 h at room temperature. After washing in PBS, the sections were incubated in
Alexa Fluor® 488 donkey anti-rabbit IgG (Cat # A21206, Invitrogen; dilution 1:500) for 2 h at room temperature, and following additional washings in PBS, sections were mounted onto SuperFrost slides, dried overnight and coverslipped with Vectashield mounting media with DAPI.

Separate series of free-floating coronal sections ranging from approximately -1.34 to -3.08 mm (Figure 1A-F) past bregma from mice carrying one Ghsr-IRES-Cre allele and one ROSA26-YFP transgene underwent immunohistochemistry for YFP. The same protocol as described above was followed except that the primary antibody used was chicken anti-GFP antibody (Cat # GFP-1010; Aves Labs, Davis, CA, dilution 1:1000) and the secondary antibody used was Alexa Fluor® 488 goat anti-chicken IgG (Cat # A11039, Invitrogen; dilution 1:500).

**Dual-label histochemistry**

Series of free-floating coronal sections ranging from approximately -1.70 to -2.06 mm past bregma from 5 mice carrying one Ghsr-IRES-Cre allele and one ROSA26-YFP transgene first underwent immunohistochemistry for YFP (Supplemental Figure 1) using RNase-free conditions. Sections were rinsed in DEPC-PBS, immersed in 0.5% Triton X-100 solution in DEPC-PBS containing 1µL RNase inhibitor (cat: 100000840; RNaseOUT™ Recombinant Ribonuclease Inhibitor; Invitrogen) per 100 µL DEPC-PBS for 30 min. Sections were incubated in diluted chicken anti-GFP antibody (Aves Labs; dilution 1:1000 + 1 µL RNase inhibitor per 100 µL DEPC-PBS) for 6 h at room temperature. After washing in DEPC-PBS, the sections were incubated in Alexa Fluor® 488 goat anti-chicken IgG (Invitrogen; dilution 1:500 + 1 µL RNase inhibitor per 100 µL DEPC-PBS) for 1 h at room temperature. Following additional washings in RNase inhibitor-treated DEPC-PBS, sections were mounted onto SuperFrost slides and processed for RNAScope *in situ* hybridization histochemistry as described above.

**Image capture and analysis**

Most images were captured with either 10X or 20X objectives of a fluorescence microscope (Leica DM6 B digital research microscope equipped with Leica DFC 9000 GT digital microscopy camera; Leica Microsystems Gmbh, Wetzlar, Germany) plus LAS X software or a laser scanning confocal microscope (Zeiss LSM 880 airyscan; Oberkochen, Baden-Württemberg, Germany) plus ZEN blue software. Single-, double-, or triple-labeled fluorescence images or laser scanning images were captured using suitable filter sets or respective lasers for AlexaFluor 488 (green), AlexaFluor 594 / mCherry (red), and DAPI (blue). A series of brain sections were captured with the fluorescence microscope and processed using LAS X software (Figure 1, 2B-G, 3C-H, 7D-G, Supplemental Figure 1-4). A series of optical slices through the MBH was captured and processed using Zeiss ZEN blue image browsing software (Figure 5C-H, 6, 8C-E, Supplemental Figure 5). Neuroanatomical co-localization between cells containing mCherry fluorescence (red) and c-fos or nNOS-immunoreactivity (green) appeared as an overlap of these two fluorophores (yellow). DAPI counterstaining (to identify nuclei) and comparisons to a mouse brain atlas (53) were used to identify the regional boundaries between different MBH regions. Cells showing clear, round DAPI-stained nuclei surrounded by mCherry were identified as being mCherry-positive. c-fos-immunoreactive cells were identified by the presence of green nuclei with DAPI counterstaining. Cells showing clear, round DAPI-stained nuclei surrounded by green fluorescence were identified as nNOS-immunoreactive cells. Manually counting of cells was performed with the assistance of the Adobe Photoshop 22.3.0 software (Adobe Systems, San Jose, CA) counting tool bilaterally for each mouse. See above for the levels of the ARC that were analyzed for c-fos and nNOS. For PMV, one level approximately -2.30 mm past bregma was analyzed. Similarly, the co-localization of mCherry with c-fos in the VMH, or mCherry with nNOS in the VMHvl were counted in each animal, and
percentage were determined. The size and brightness of all the captured photomicrographs were adjusted uniformly with Adobe Photoshop.

To compare Ghsr mRNA expression between sedentary and HIIE-exposed C57BL/6N mice, images of coronal MBH sections from those mice were stained for Ghsr mRNA by RNAscope (red) and with DAPI counterstaining (blue; to identify nuclei) and then were compared to a mouse brain atlas (53) to identify the boundaries between different MBH and nearby regions. No adjustments to intensity or exposure were made to the images. Boundaries for the ARC, VMH, and DMH were determined for each individual section and manually drawn on the images using the freehand tool in Adobe Photoshop. The ARC and VMH were analyzed bilaterally at three different distances from bregma (-1.34 mm, -1.82 mm, and -2.06 mm), while the DMH was analyzed bilaterally at two different distances from bregma (-1.82 mm and -2.06 mm), using ImageJ software (NIH; https://imagej.nih.gov/ij/): Images were exported into ImageJ and converted to 8-bit gray scale to create a monochromatic image. A threshold for the red channel was set between 24 (min) and 100 (max). Afterwards, with the assistance of the ImageJ ‘Analyze’ and “Measure” tools, the following parameters were determined: area, area faction, integrated density, and minimum + maximum gray values. The % fluorescent area of Ghsr expression on each side of the ARC was determined for each of the three coronal levels described above and then averaged together for each animal. The same was done for the VMH (at 3 levels) and DMH (at 2 levels). The data were averaged for the sedentary mice (n=4) and separately, for the HIIE-exposed mice (n= 5).

Statistics
Data are presented as mean ± SEM and are analyzed by paired student’s t-test, unpaired student’s t-test, one-way ANOVA, or two-way ANOVA, as indicated in the Figure legends. Šidák post hoc testing was used to further investigate differences if significant ANOVA effects were found. Correlations were done by Pearson’s correlation and simple linear regression analysis. All data were analyzed using GraphPad Prism version 9.0.2 (GraphPad Software, Inc., San Diego, CA). No outliers were detected by Grubb’s test. P-values < 0.05 were considered statistically significant.

Data availability
Values for all data points shown in graphs and behind any reported means are available upon request from the corresponding author.

Author Contributions
O.S. and J.M.Z. developed the concept; O.S. and J.M.Z. developed the experimental strategy. O.S., S.B.O., S.V., K.S., D.G., S.P. performed the experiments. S.O.L worked with C.P.R., N.P.M., and C.L to organize the breeding schedule and all aspects of animal husbandry required for the study. O.S. and J.M.Z. analyzed the data. J.M.Z. secured funding for the project. L.L.M. helped to edit the paper and troubleshoot experimental protocols. O.S and J.M.Z. wrote the paper.

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Endocrinology, in Honor of Daniel Foster, M.D. (to J.M.Z.), and the Peter O'Donnell Jr. Brain Institute (OBI) Sprouts Program (to O.S.).

References


Table 1. Cre-dependent mCherry expression in different brain regions of hM4Di-injected Ghsr-IRES-Cre mice characterized as “hits” or “misses,” with expression subjectively ranked from undetectable (-) to plenty (+++).

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**Abbreviation:**
ARC - Arcuate nucleus; DMH - Dorsomedial hypothalamic nucleus; DTM - Dorsal tuberomammillary nucleus; EW - Edinger-Westphal nucleus; LM - lateral mamillary nucleus medial part; ML - medial mamillary nucleus, lateral part; MM - medial mamillary nucleus; MTu - median tuberal nucleus; PH - posterior hypothalamic area; PMD - Premammillary nucleus, dorsal part; PMV - Premammillary nucleus, ventral part; RChL - Retrochiasmatic area, lateral part; SuM - Supramammillary nucleus; VMHc - Ventromedial hypothalamic nucleus, central aspect; VMHcap - Ventromedial hypothalamic nucleus, capsular region; VMHdm - Ventromedial hypothalamic nucleus, dorsomedial aspect; VMHvl - Ventromedial hypothalamic nucleus, ventrolateral aspect.
Figure 1. Confirmation of expected Cre recombinase activity within GHSR-expressing neurons of Ghsr-IRES-Cre mice. A-F, Low magnification fluorescence photomicrographs showing YFP-immunoreactive cell bodies (green) in coronal MBH and midbrain sections of a representative Ghsr-IRES-Cre X ROSA26-YFP reporter mouse. G-L, Low magnification fluorescence photomicrographs showing Ghsr mRNA expression (red), as determined using RNAscope in situ hybridization histochemistry in coronal MBH and midbrain sections of a representative NPY-GFP mouse. M-O, Low magnification fluorescence photomicrographs
showing expression of Ghsr mRNA (red), GFP (green), and their co-localization (yellow) in coronal MBH sections of a representative NPY-GFP mouse. Scale bars = 100 µm in A-O. Approximate distance of each coronal section from bregma (B) is indicated in the lower left corner of each panel. v - third ventricle; ARC - Arcuate nucleus; DMH - Dorsomedial hypothalamic nucleus; DTM - Dorsal tuberomammillary nucleus; EW - Edinger-Westphal nucleus; LM - lateral mammillary nucleus; MM - medial mammillary nucleus, medial part; ML - medial mammillary nucleus, lateral part; PMD - Premammillary nucleus, dorsal part; PMV - Premammillary nucleus, ventral part; PVH - Paraventricular hypothalamic nucleus; RChL - Retrochiasmatic area, lateral part; SN - Substantia nigra; SuML - Supramammillar nucleus, lateral part; SuMM - Supramammillary nucleus, medial part; VMHc - Ventromedial hypothalamic nucleus, central aspect; VMHcap - Ventromedial hypothalamic nucleus, capsular region; VMHdm - ventromedial hypothalamic nucleus, dorsomedial aspect; VMHvl - Ventromedial hypothalamic nucleus, ventrolateral aspect; VTA - Ventral tegmental area.
Figure 2. High intensity interval exercise (HIIE) increases hypothalamic Ghsr expression. **A**, Schematic of the experimental design used to generate the data in this figure. **B-G**, Low magnification fluorescence photomicrographs showing Ghsr mRNA expression (red), as determined using RNAscope in situ hybridization histochemistry in coronal MBH sections of a representative sedentary (sed) mouse (**B-D**) and a representative mouse that underwent HIIE (**E-G**). DAPI (blue) is used as counterstaining. The approximate distances of each coronal section from bregma (B) are indicated in the lower left corner of each panel. Scale bars = 50 µm in **B-G**. v - third ventricle; ARC - Arcuate nucleus; DMH - Dorsomedial hypothalamic nucleus; PVH - Paraventricular hypothalamic nucleus; RChL - Retrochiasmatic area, lateral part; VMHc - Ventromedial hypothalamic nucleus, central aspect; VMHcap - Ventromedial hypothalamic nucleus, capsular region; VMHdm - Ventromedial hypothalamic nucleus, dorsomedial aspect; VMHvl - Ventromedial hypothalamic nucleus, ventrolateral aspect. **H-J**, Effects of HIIE on the % fluorescent area of GHSR expression in ARC. **K**, Changes in blood glucose (mg/dL). **L**, Changes in blood lactate (mmol/L). **M**, Changes in body weight (g).
representing Ghsr expression within the (H) ARC, (I) VMH, and (J) DMH. K-L, Effects of HIIE on (K) blood glucose and (L) blood lactate. (M) Body weights of mice that were tested here (sedentary mice, n= 4 vs. mice that underwent HIIE, n= 5). Data were analyzed by unpaired Student’s t test. Statistically significant differences are indicated by asterisks: *p<0.05, **p<0.01, ***p<0.001, ns - no statistically significant difference.
Figure 3
Figure 3. Inhibition of GHSR-expressing MBH neurons attenuates food intake after HIIE. 

A, Schematic of coronal brain section demonstrates the sites of injection of AAV-hSyn-DIO-hM4D(Gi)-mCherry virus (hM4Di) within the MBH of Ghsr-IRES-Cre mice. B, Schematic of the experimental design used to generate the data in this figure. C-H, Representative coronal brain sections from Ghsr-IRES-Cre mice injected with the virus, demonstrating Cre-dependent mCherry expression in the MBH (red), or the lack thereof. DAPI (blue) is used as counterstaining. The approximate distances of each coronal section from bregma (B) are indicated in the lower left corner of each panel. C-E, The top row displays brain sections from a representative mouse classified as a "hit" as a result of a correctly-targeted MBH. F-H, The bottom row displays brain sections from a representative mouse classified as a "miss" as a result of incorrect targeting of the virus. Scale bar = 100 μm in C-H. I-L, Effects of administration of CNO (0.3 mg/kg BW, i.p.) vs. saline in “hits” on (I) food intake, (J) blood glucose, (K) blood lactate, and (L) body weight. M-P, Effects of administration of CNO (0.3 mg/kg BW, i.p.) vs. saline in “misses” on (M) food intake, (N) blood glucose, (O) blood lactate, and (P) and body weight. n= 16 “hits” and n= 11 “misses”. Data in panels I and M were analyzed by repeated measures two-way ANOVA followed by Šidák post hoc multiple comparisons test. Data in panels J-L and N-P were analyzed by paired Student’s t test. Statistically significant differences are indicated by asterisks: *p<0.05; ns - no statistically significant difference. v - third ventricle; ARC - Arcuate nucleus; DMH - Dorsomedial hypothalamic nucleus; DTM - Dorsal tuberomammillary nucleus; PMD - Premammillary nucleus, dorsal part; PMV - Premammillary nucleus, ventral part; VMHc - Ventromedial hypothalamic nucleus, central aspect; VMHcap - Ventromedial hypothalamic nucleus, capsular region; VMHdm - ventromedial hypothalamic nucleus, dorsomedial aspect; VMHvl - Ventromedial hypothalamic nucleus, ventrolateral aspect.
Figure 4. Inhibition of GHSR-expressing MBH neurons impairs exercise endurance. A, Schematic of the experimental design used to generate the data in this figure. B-G, Effects of administration of CNO (0.3 mg/kg BW, i.p.) vs. saline in AAV-hSyn-DIO-hM4D(Gi)-mCherry virus (hM4Di)-injected “hits” on (B) maximal running distance, (C) total running duration, (D) maximal running speed, (E) blood glucose at exhaustion, (F) blood lactate at exhaustion, and (G) plasma ghrelin at exhaustion. H-M, Effects of administration of CNO (0.3 mg/kg BW, i.p.) vs. saline in hM4Di-injected “misses” on (H) maximal running distance, (I) total running duration, (J) maximal running speed, (K) blood glucose at exhaustion, (L) blood lactate at exhaustion, and (M) plasma ghrelin at exhaustion. n= 16 “hits” and n= 11 “misses”. Data were analyzed by paired Student’s t test. Statistically significant differences are indicated by asterisks: *p<0.05, **p<0.01, ****p<0.0001, ns - no statistically significant difference.
Figure 5
Figure 5. Inhibition of GHSR-expressing MBH neurons impairs exercise endurance and c-fos induction in the MBH following an exercise endurance test. A, Schematic of coronal brain section demonstrates the sites of injection of AAV-hSyn-DIO-hM4D(Gi)-mCherry virus (hM4Di) or control AAV-hSyn-DIO-mCherry (“Cre-dependent mCherry control”) virus within the MBH of Ghsr-IRES-Cre mice. B, Schematic of the experimental design used to generate the data in this figure. C-H, Confocal images of coronal brain sections showing c-fos-immunoreactivity (green) and mCherry expression (red) in the MBH and adjacent regions of a representative “Cre-dependent mCherry control virus”-injected “hit” (C-E) and in a representative hM4Di-injected “hit” (F-H) sacrificed at the time of exhaustion. DAPI, which is used as counterstaining, is shown in blue. Scale bar in H = 100 μm and applies to panels C-H. Approximate distance of the coronal section from bregma (B) is indicated in the lower left corner of panels C-H. I-M, Effects of administration of CNO (0.3 mg/kg BW, i.p.) in hM4Di “hits” (n=5) vs. Cre-dependent mCherry control “hits” (n=4) on I, maximal running distance, J, total running duration, K, maximal running speed, L, blood glucose, M, blood lactate, in exercised mice at exhaustion. N-O, Correlations between N, plasma ghrelin, and O, blood glucose, with distance run during the exercise endurance test in CNO-treated hM4Di “hits” (n = 21, which includes the 5 hM4Di “hits” from this study and the 16 “hits” from Fig. 4). P-R, Numbers of c-fos-immunoreactive cells in the P, ARC and Q, VMH of hM4Di “hits” (n=5) vs. Cre-dependent mCherry control “hits” (n =4) following CNO and the exercise endurance test. R, % co-localization of c-fos positive cells with mCherry in VMH, hM4Di “hits” (n=5) vs. Cre-dependent mCherry control “hits” (n =4) following CNO and the exercise endurance test. Data were analyzed by unpaired Student’s t test (I-M, P-R) or by Pearson’s correlation and simple linear regression analysis (N-O), whereby Pearson’s correlation coefficient (r) and p values are indicated in the figure panels and whereby the solid lines represent the fitted linear regression curves and the dotted lines represent the s.e.m. Statistically significant differences are indicated by asterisks: *p<0.05, **p<0.01, ***p<0.001.
Figure 6. Overlap of nNOS and Cre-dependent mCherry expression within the MBH of an hM4Di-injected Ghsr-IRES-Cre “hit”. A-D, Low and E-H, High magnification confocal images of a coronal brain section (approximately -1.94 mm from Bregma) from a representative Ghsr-IRES-Cre mouse correctly-targeted with an injection of hM4Di into the MBH. This mouse was sacrificed at the time of exhaustion after having received CNO and then being submitted to the exercise endurance protocol schematized in Fig. 5B. A,E, nNOS-immunoreactivity (green). B,F, mCherry fluorescence (red). C,G, Merged images to demonstrate co-expression of nNOS and mCherry (yellow). D,H, Merged images with DAPI counterstain in blue. Arrows point to neurons that exclusively express mCherry (correctly-targeted GHSR-expressing neurons). Arrowheads point to neurons that co-express mCherry + nNOS. A total of n=3 cases were analyzed. Scale bar in D = 100 μm and applies to A-D. Scale bar in H = 100 μm and applies to E-H. v - third ventricle; ARC - Arcuate nucleus; DMH - Dorsomedial hypothalamic nucleus; LH - Lateral hypothalamic area; VMHc - Ventromedial hypothalamic nucleus, central aspect; VMHcap - Ventromedial hypothalamic nucleus, capsular region; VMHdm - ventromedial hypothalamic nucleus, dorsomedial aspect; VMHvl - Ventromedial hypothalamic nucleus, ventrolateral aspect.
Figure 7. Inhibition of GHSR-expressing MBH neurons reduces food intake and MBH c-fos induction in response to administered ghrelin. 

A, Schematic of the experimental design used to generate the data in this figure. 

B-C, Effects of administration of CNO (0.3 mg/kg BW, i.p.) in AAV-hSyn-DIO-hM4D(Gi)-mCherry virus (hM4Di)-injected Ghsr-IRES-Cre "hits" vs "misses" on B, food intake and C, c-fos induction within the ARC measured 2h following delivery of ghrelin (1 mg/kg BW s.c.). 

D-G, Fluorescence images of coronal brain sections showing c-fos-immunoreactivity (green) and mCherry expression (red) in the MBH of a representative hM4Di-injected “hit” (D-E) and a representative hM4Di-injected “miss” (F-G) sacrificed 2h following ghrelin delivery. DAPI counterstaining is shown in blue. Scale bar in G = 50 μm and applies to panels D-G. Approximate distance of the coronal section from bregma (B) is indicated in the lower left corner of panels. 

n = 16 “hits” and n = 11 “misses” were used for food intake measurements. n = 5 “hits” and n = 5 “misses” were used for quantification of c-fos induction. Data in panels (B-C) were analyzed by unpaired Student’s t test. Statistically significant differences are indicated by asterisks: **p<0.01, ****p<0.0001. v - third ventricle; ARC - Arcuate nucleus; DMH - Dorsomedial hypothalamic nucleus; VMHc - Ventromedial hypothalamic nucleus, central aspect; VMHdm - ventromedial hypothalamic nucleus, dorsomedial aspect; VMHvl - Ventromedial hypothalamic nucleus, ventrolateral aspect.
**Figure 8.** Inhibition of GHSR-expressing MBH neurons does not affect glucose tolerance.

**A,** Schematic of coronal brain section demonstrates the sites of injection of AAV-hSyn-DIO-hM4D(Gi)-mCherry virus (hM4Di), control AAV-hSyn-DIO-mCherry (“Cre-dependent mCherry control”) virus, or AAV-hSyn-mCherry (“non-Cre-dependent mCherry control”) virus within the MBH of *Ghsr-IRES-Cre* mice. **B,** Schematic of the experimental design used to generate the data in this figure. **C-E,** Confocal images of coronal brain sections showing mCherry expression (red) in the MBH of a representative **C,** “non-Cre-dependent mCherry control virus”-injected “hit”, **D,** “Cre-dependent mCherry control virus”-injected “hit”, and **E,** hM4Di-injected “hit”. DAPI counterstaining is shown in blue. Scale bar in **E** = 100 μm and applies to panels **C-E.** Approximate distance of the coronal section from bregma (B) is indicated in the lower left corner of panels **C-E.** **F-H,** Effects of administration of CNO (0.3 mg/kg BW, i.p.) in “non-Cre-dependent mCherry control virus”-injected “hits” (n= 4), “Cre-dependent mCherry control virus”-injected “hits” (n= 4) and hM4Di-virus-injected “hits” (n= 5) on the following measurements obtained as part of an oGTT: **F,** blood glucose curves assessed over the first 120 min following administration of glucose (2 mg/kg BW) by oral gavage, **G,** fasting blood glucose levels at the start of the oGTT (after a 6 h fast and just prior to glucose administration), and **H,** blood glucose...
area under curve. Data in panel (F) were analyzed by repeated measures two-way ANOVA followed by Šidák post hoc multiple comparison test. Data in panels (G-H) were analyzed with One-way ANOVA with Šidák multiple comparisons test. v - third ventricle; ARC - Arcuate nucleus; DMH - Dorsomedial hypothalamic nucleus; LH - Lateral hypothalamic area; VMHc - Ventromedial hypothalamic nucleus, central aspect; VMHcap - Ventromedial hypothalamic nucleus, capsular region; VMHdm - ventromedial hypothalamic nucleus, dorsomedial aspect; VMHvl - Ventromedial hypothalamic nucleus, ventrolateral aspect.