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Ghrelin infused into the dorsomedial hypothalamus of male mice increases food intake and adiposity.

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ABSTRACT

Ghrelin is a 28 amino acid peptide hormone that targets the brain to promote feeding and adiposity. The ghrelin receptor, the GHSR1a, is expressed within most hypothalamic nuclei, including the DMH, but the role of GHSR1a in this region on energy balance is unknown. In order to investigate whether GHSR1a within the DMH modulate energy balance, we implanted osmotic minipumps filled with saline, ghrelin, or the GHSR1a antagonist JMV2959, and connected it to a cannula aimed unilaterally at the DMH of adult male C57BLJ6 mice and assessed their metabolic profile. We found that chronic infusion of ghrelin in the DMH promoted an increase in caloric intake as well as a decrease in energy expenditure. This translated to an overall increase in weight gain, primarily in the form of adipose tissue in ghrelin treated animals. Further, chronic ghrelin unilateral infusion into the DMH slowed glucose clearance. These results suggest that GHSR in the DMH significantly contribute to the metabolic effects produced by ghrelin.

1. Introduction

The orexigenic peptide hormone ghrelin plays an important role in energy metabolism by regulating food intake, body weight, and glucose homeostasis. Ghrelin is produced by the stomach in conditions of negative energy state and prior to scheduled meals, and is released into the circulation to stimulate feeding behaviour, increase carbohydrate metabolism and decrease fat utilization, ultimately increasing fat deposition [1, 2]. Peripheral administration of ghrelin increases food intake and body weight, preserves white adipose tissue (WAT), promotes the preferential burning of carbohydrates for energy, and reduces glucose clearance [1-4]. The receptor for ghrelin, the growth hormone secretagogue receptor (GHSR1a), is expressed in many peripheral and central tissues, and in particular is expressed in abundance within the hypothalamus [5–7]. Central administration of ghrelin potently stimulates food intake, even in satiated animals, and these orexigenic effects have been associated by the actions of ghrelin on neuropeptide y (NPY)/agouti related peptide (AGRP) neurons in the arcuate nucleus (ARC) [1, 2, 8–10].

The DMH has been implicated in the regulation of energy homeostasis, and is known to play a role in the modulation of circadian rhythms, food intake and body weight, sympathetic activity and the stress response, as well as in body temperature and thermogenesis [11–16]. Lesions of the DMH lead to diminished food and water intake, as well as reductions in body weight and growth [11], suggesting it plays an important role in multiple aspects of energy regulation. Interestingly, chronic food restriction or intraperitoneal injections of ghrelin produce a marked increase in FOS immunoreactivity in the DMH [17, 18]. These increases in cell activation in the DMH may be due to indirect effects of ghrelin on NPY/AGRP neurons in the ARC, which have direct projections to the DMH [19]. Nevertheless, the DMH also shows relatively high levels of GHSR1a mRNA expression suggesting that ghrelin acts directly in the DMH, but little is known about the physiological function of GHSR1a expression in this nucleus. Selective knockdown of the GHSR1a in the DMH of *ad libitum* rats attenuates running wheel activity but does not affect food intake or body weight [20]. In the current experiment we examined how chronically blocking or stimulating the GHSR1a in the DMH of mice affects metabolic outcome, including food intake, body weight and body composition, glucose clearance, and energy expenditure.

2. Experimental procedures

2.1. Animals

All procedures were approved by the Carleton University Animal Care Committee according to the guidelines of the Canadian Council of Animal Care (CCAC). Male mice (C57BL/J6, 20-25 grams) were

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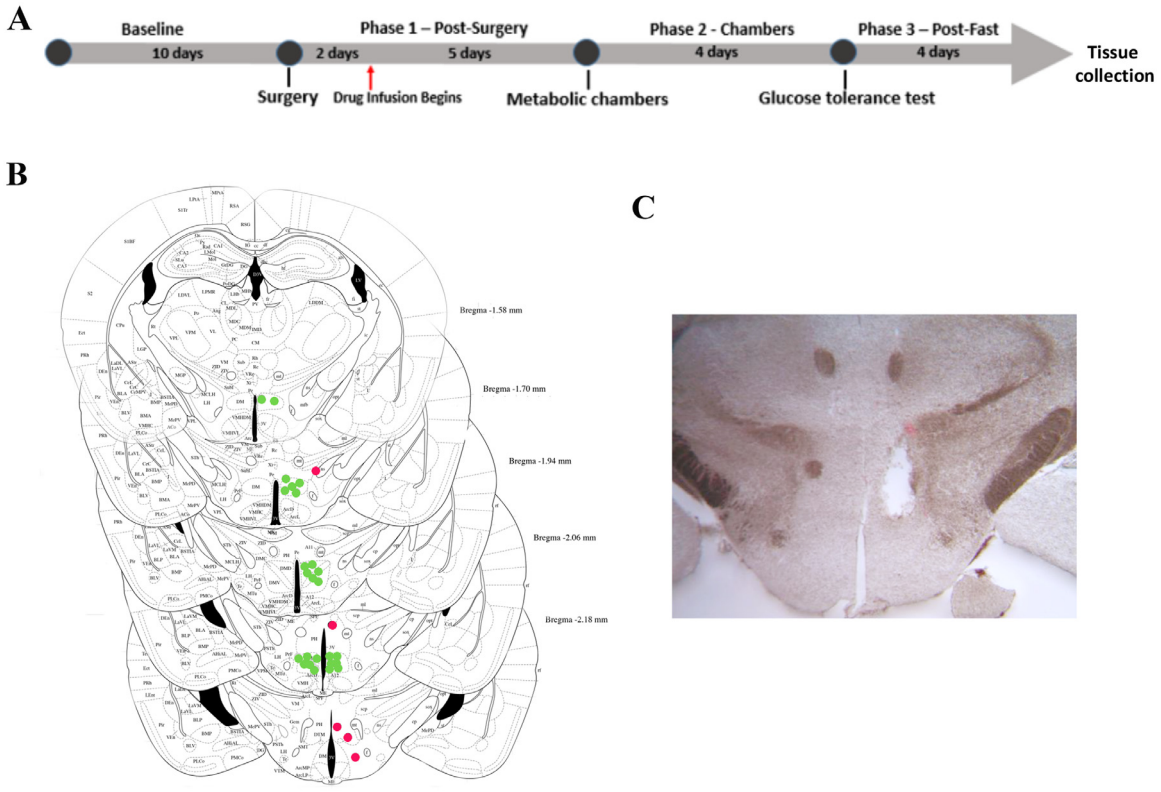

Fig. 1. Experimental timeline and cannulae placements for all experimental animals. (A) Depiction of experimental timeline from baseline to the end of the drug delivery period. (B) Coronal sections adapted from the Paxinos and Franklin Mouse Brain Atlas [23], depicting the DMH from anterior to posterior hypothalamus. The red dots represent mice with placements falling outside the DMH and that were therefore excluded from all analyses. The green dots represent those placements in mice that fall within or just above the DMH and were therefore included in the analysis. (C) Image taken of representative cannula placement for location within the dorsomedial hypothalamus.

obtained from Charles Rivers farms (St. Constant, Quebec). Figure 1A depicts the experimental timeline followed in this study. All mice were individually housed in clear plexiglass cages with disposable rodent home and nesting material provided as enrichment, at a temperature of 20 °C and humidity of 40%. All mice had a*d libitum* access to chow (2.9 kcal/g, with 70% of calories derived from carbohydrates) and tap water throughout the experiment. In addition, a high-fat diet containing 60% of calories from fat (TD 06414, Harlan; 5.2 kcal/g) was supplied between 8:30 a.m. and 12:30 p.m. each day. As shown in Fig. 1A, baseline food intake and body weight were measured for 10 days to acclimate the animals to the high fat diet and to obtain reliable pre-treatment data. Following the baseline period, mice were assigned to one of three experimental drug treatment groups: vehicle (n = 12), ghrelin (n = 12; 10 μg/day in isotonic saline; Peptides International), JMV2959 (n = 12; 20 μg/day in isotonic saline; Calbiochem©). All mice underwent surgery to implant an intracranial cannula attached to an osmotic minipump, delivering the drug of their assigned group. Surgical procedures were based on those described previously by Patterson et al. [21]. Prior to surgery, all mice were anesthetized using 4% isoflurane mixed with oxygen and secured into a mouse stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Surgiprep and Priviodine were applied to the scalp to provide sterilization, and tear gel was applied to the eyes to prevent dehydration. A midline incision was made, and the skin was withdrawn to allow for a clear view of bregma. A 28-gauge stainless steel unilateral cannula (Alzet Brain Infusion Kit) coupled via a polyethylene catheter to a filled osmotic minipump (Alzet Mini-Osmotic Pump – Model 1004; flow rate 0.11 μL/h for 28 days) was implanted into the dorsomedial hypothalamus. Stereotactic coordinates relative to bregma were: AP 1.6 mm, ML 0.4 mm, and DV 5.25 mm using the Paxinos and Franklin Mouse Brain Atlas [22]. Dental cement was applied to secure the implant. The minipump was subcutaneously implanted after separating the skin from the muscle using blunt dissection. Silk surgical sutures were used to close the incision, and Polysporin and Lidocaine were administered to the surgical site to prevent bacterial infection and limit pain. Mice were then allowed to recover in a clean cage supplied with a heating pad, and Meloxicam (2 mg/kg) was injected subcutaneously once per day for three days to provide postoperative analgesia. Mice were closely monitored for the next week, and daily weight, high fat diet and chow intake measurements continued. Because the osmotic minipump was not primed prior to insertion, and the polyethylene catheter was filled with the same drug as the minipump, drug infusion began 48 h after surgery.

2.2. Metabolic chambers

At least one week following surgery, mice were moved to a separate room and housed individually in phenomaster/labmaster metabolic cages (TSE instruments, Chesterfield, Missouri) for a period of 48 h under the same feeding conditions as in their home cage. While in the metabolic cage, TSE software records oxygen consumption (VO_2), carbon dioxide production (VCO_2), food intake, water intake, and locomotor activity every 30 min for 48 h. Respiratory exchange ratio (RER) is the ratio of the amount of VCO_2 produced to the amount of O_2 consumed, and is calculated by the software. Energy expenditure (kcal/h/kg) is based on the caloric value (CV) of RER, oxygen consumption, and body weight of the animal: $\frac{CV\,(of\ RER) * VO2(\frac{ml}{h})}{body\ weight\ (kg)}$ and is also calculated by the software. Only the last 24 h of this period were used for analyses, as the first 24 h are typically used to allow acclimation. Upon the completion of this test, mice were returned to their home cages.

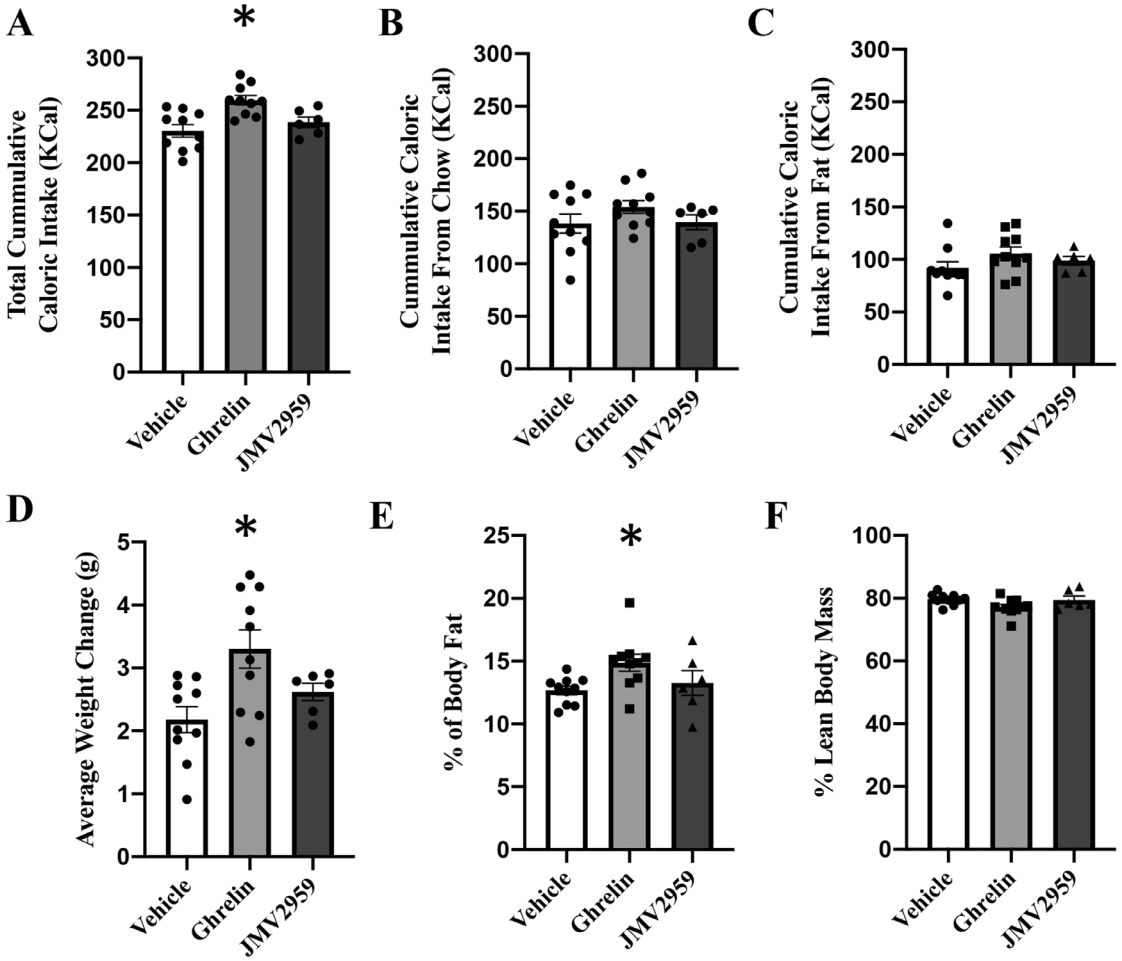

Fig. 2. Average cumulative caloric intake, body weight change, and body composition measures across the duration of experiment in mice infused with vehicle, ghrelin or JMV2959 into the DMH. As seen in this image, ghrelin infused mice increased their total caloric intake without affecting diet preference A,B &C). These increase was associated with an increase in weight change (D) and increase in percent body fat mass (E) but not lean mass (Fig. 1F). All data are expressed as mean +/− SEM. * $p < 0.05$.

2.3. Glucose tolerance test

Mice underwent a glucose tolerance test at least 96 h after they were tested in the metabolic cages to determine the rate of glucose clearance from blood. Prior to the test, mice were transferred to clean cages and fasted overnight, with access to water *ad libitum*. The following morning, immediately prior to intraperitoneal injection of glucose, a small incision was made at the tip of the mouse's tail with sterile scissors. A Contour Next blood glucose test strip was inserted into a Contour Next meter, and a small drop of blood from the tail was placed onto the strip and glucose levels were measured by the meter. Glucose was then injected intraperitoneally at a dose of 2g of glucose per kg of body mass. Blood was taken at 15, 30, 60, and 120 min after injection and glucose was measured in the same manner. To prevent further blood loss from the incision, pressure was applied briefly to the incision after each measurement. Upon completion of the entire test, food was returned to the animals.

At least 2 days after GTT, after all testing was completed, mice were sacrificed by rapid decapitation to obtain blood and tissue samples. Brains were prepared for histological evaluation and confirmation of cannula placements, and body composition was measured from the carcasses using an EchoMRI Body Composition Analyzer EF-020 (Houston, Texas, www.echomri.com).

2.4. Cannula placements

Brains were extracted and stored in 4% PFA in PB for at least 72 h in order to allow for full fixation of the tissue. They were then removed from the PFA and transferred to a 30% sucrose solution in PB (with sodium azide), in order to cryoprotect the brains before slicing. Once the brains sank to the bottom of the vial, they were sliced at 40 μm using a cryostat, and tissue sections were mounted onto slides. Once dry, sections were viewed under a microscope for verification of correct cannula placements. Data from mice with incorrect cannula placements were removed from all statistical analyses.

2.5. Statistical Analyses

Food intake was analyzed as total cumulative food intake over the period of infusion using a one-way ANOVA with drug treatment (saline, ghrelin, or JMV2959) as the independent variable. Body weight gain was calculated as the difference between the weight on the last day of infusion subtracted from the weight on the first day of drug infusion and analyzed using a one-way ANOVA with drug treatment as the independent variable. Furthermore, in order to account for changes in total caloric intake or body weight, measures of energy expenditure and respiratory exchange ratio, we used ANCOVAs with lean mass, total caloric intake, or chow intake as a covariate. Glucose levels across the test were analyzed by repeated measures ANOVAs, with time as the

within subjects factor, and drug treatment as the between-subjects factor. Where appropriate, Tukey's HSD pairwise comparisons were used. Light and dark phase of metabolic measures, body composition, and area under the curve for glucose levels were analyzed by univariate ANOVAs, and Tukey's HSD post-hoc analyses were performed where appropriate.

3. Results

3.1. Cannula placements

Three mice did not survive the surgery and their baseline data were removed from the analyses. Six additional mice were removed from the data analyses due to incorrect cannula placements, and one mouse was removed because of abnormal cage behavior. Thus, 26 animals were included in the final analyses, with the following group numbers: saline (n = 10), ghrelin (n = 10), JMV2959 (n = 6). Fig. 1B depicts final cannula placements.

3.2. Ghrelin delivery in the DMH increases caloric intake, weight gain and adiposity

Fig. 2 shows the effects of vehicle, ghrelin and JMV2959 on caloric intake, weight gain, and body composition (see Fig. 2). Statistical analyses of the cumulative food intake data revealed that intra-DMH ghrelin promoted food intake, as seen by a significant increase in cumulative total caloric intake in ghrelin vs both saline and JMV2959 treated animals ($F(2,28) = 8.94$, $p = 0.001$; Tukeys posthoc tests; see Fig. 2A). This effect was not driven by the intake of a specific diet (i.e. either chow or high fat, see Fig. 2B and C, respectively; $p > 0.05$), but by the combined increase in calories from both high fat diet and chow in ghrelin treated animals.

Chronic infusions of ghrelin also resulted in a significant increase in average weight change compared to vehicle treated mice ($F(2,23) = 5.74$, $p = 0.009$; Tukey's posthoc test). JMV2959 treated mice did not differ in weight change from either saline or ghrelin treated mice ($p > 0.05$, see Fig. 2D). Body composition analyses showed that ghrelin treatment increased the proportion of body fat compared to vehicle treated mice ($F(2,23) = 3.6$, $p = 0.04$; Tukey's posthoc test), but not JMV2959 treated mice (See Fig. 2E). Although ghrelin treatment appeared to reduce the proportion of lean mass, this effect was not statistically significant ($F(2,23) = 2.96$, $p = 0.072$; see Fig. 2F).

3.3. Ghrelin delivery in the DMH reduces energy expenditure

All animals were housed in phenomaster/labmaster metabolic cages for 48 h to assess the effect of drug treatment on fuel utilization patterns. Fig. 3 depicts energy expenditure (Fig. 3A), respiratory exchange ratio (RER, Fig. 3B) and locomotor activity (Fig. 3C) over the last 24 h spent in the indirect calorimetry boxes.

One way ANOVAs followed by Tukey's post hoc tests conducted on the total average energy expenditure measures for those 24 h showed that chronic ghrelin delivery into the DMH resulted in a decrease in average 24 h energy expenditure compared to energy expenditure of vehicle treated mice ($F(2.23) = 4.74$, $p = 0.019$; See Fig. 3D). The effects of ghrelin on energy expenditure were more evident during the light phase of the cycle as demonstrated by a one way ANOVA followed by Tuckey's posthoc tests ($F(2.23) = 4.19$, $p = 0.028$). A one way ANOVA on the average energy expenditure during the dark phase was also significant ($F(2.23) = 3.8$, $p = 0.038$). Posthoc Tukey's tests revealed that both ghrelin and JMV2959 tended to reduce energy expenditure during the dark phase, but these effects were not statistically significant ($p > 0.05$; Fig. 3D).

In contrast, one way ANOVAs conducted on RER measures determined that there were no overall significant differences in average 24 h RER measures ($F(2,23) = 2.98$, $p = 0.07$; See Fig. 3E). However, RER of mice receiving JMV2959 was significantly higher than RER of vehicle or ghrelin treated mice during the light phase of the cycle ($F(2,23) = 4.95$, $p = 0.0162$, Tuckey's). This effect was not significant during the dark phase of the cycle.

Differences in energy expenditure and/or RER were independent of changes in locomotor activity, as neither chronic ghrelin nor JMV2959 significantly influenced locomotor activity across the light/dark cycle ($p > 0.05$).

3.4. JMV2959 infusions into the DMH increase glucose clearance

Fig. 4 depicts results from the glucose tolerance tests conducted on mice treated with vehicle, ghrelin or JMV2959. As shown in Fig. 4A, and as determined by a repeated measures ANOVA with time as the within subjects variable and treatment as the between groups variable, there was a significant main effect for treatment ($F(2,22) = 4.32$, $p = 0.026$) but no significant interaction effect ($p > 0.05$). Post posthoc tests (Dunnett) failed to detect overall significant differences between groups ($p > 0.05$). Nevertheless, a significant one way ANOVA followed by Tukey's posthoc tests showed that JMV2959 treated mice had lower area under the curve in terms of their glucose response to the glucose tolerance test than ghrelin treated mice ($F(2, 23) = 4.77$, $p = 0.019$). While ghrelin seemed to increase area under the curve, and JMV2959 seemed to decrease area under the curve, neither were significantly different from the area under the curved observed in vehicle treated mice ($p > 0.05$; See Fig. 4B).

4. Discussion

The DMH is an important hypothalamic region for the integration of endocrine, circadian, and neural signals to regulate food intake, energy expenditure and glucose homeostasis [11, 12, 14, 16]. Importantly, the DMH shows relatively dense expression of the GHSR1a, suggesting that ghrelin can bind directly to the DMH and affect these functions [7]. In this study we attempted to determine the effects of ghrelin stimulation or blockade of the GHSR1a in the DMH on food intake, metabolism, and glucose utilization.

As expected, unilateral intra-DMH ghrelin treatment increased caloric intake, body weight, and fat mass compared to mice treated with vehicle or mice treated with the GHSR1a antagonist JMV2959. The effects of ghrelin on the DMH occur potentially in two ways. One way these effects may be produced is by action of ghrelin on pre-synaptic terminals originating from neurons in the ARC [19]. Cells in the ARC that secrete NPY and AGRP respond to ghrelin and are important in mediating the orexigenic effects of ghrelin [19, 23, 24]. These neurons have a strong projection field to the DMH where they stimulate cells containing NPY and melanocortin receptors to modulate feeding and metabolic rate [19, 23, 24]. Because the GHSR1a is expressed in NPY/AGRP neurons, direct delivery of ghrelin may alter the activity of DMH neurons via pre-synaptic mechanisms. It is also likely that ghrelin directly targets DMH neurons given that these express GHSR1a through neuronal projections that alter feeding and energy expenditure and that may involve the activation of local hypothalamic circuits including DHM-PVN and DMH-ARC projections [16, 25], or extra-hypothalamic projections from the DMH [25, 26].

In addition to food intake, unilateral chronic ghrelin delivery into the DMH also increased weight gain and adiposity compared to mice receiving vehicle or JMV2959 infusions. While the overall increase in food intake that is produced by ghrelin being infused into the DMH probably contributes to the weight gain observed in these mice, it is also likely that ghrelin increases weight gain and adiposity through changes in metabolic rate. Indeed, ghrelin delivery into the DMH also resulted in a significant decrease in energy expenditure compared to saline treated mice. Thus, it is likely that the increases in weight gain and adiposity result from both increased caloric intake and decreased energy expenditure. Indeed, chronic peripheral ghrelin delivery results

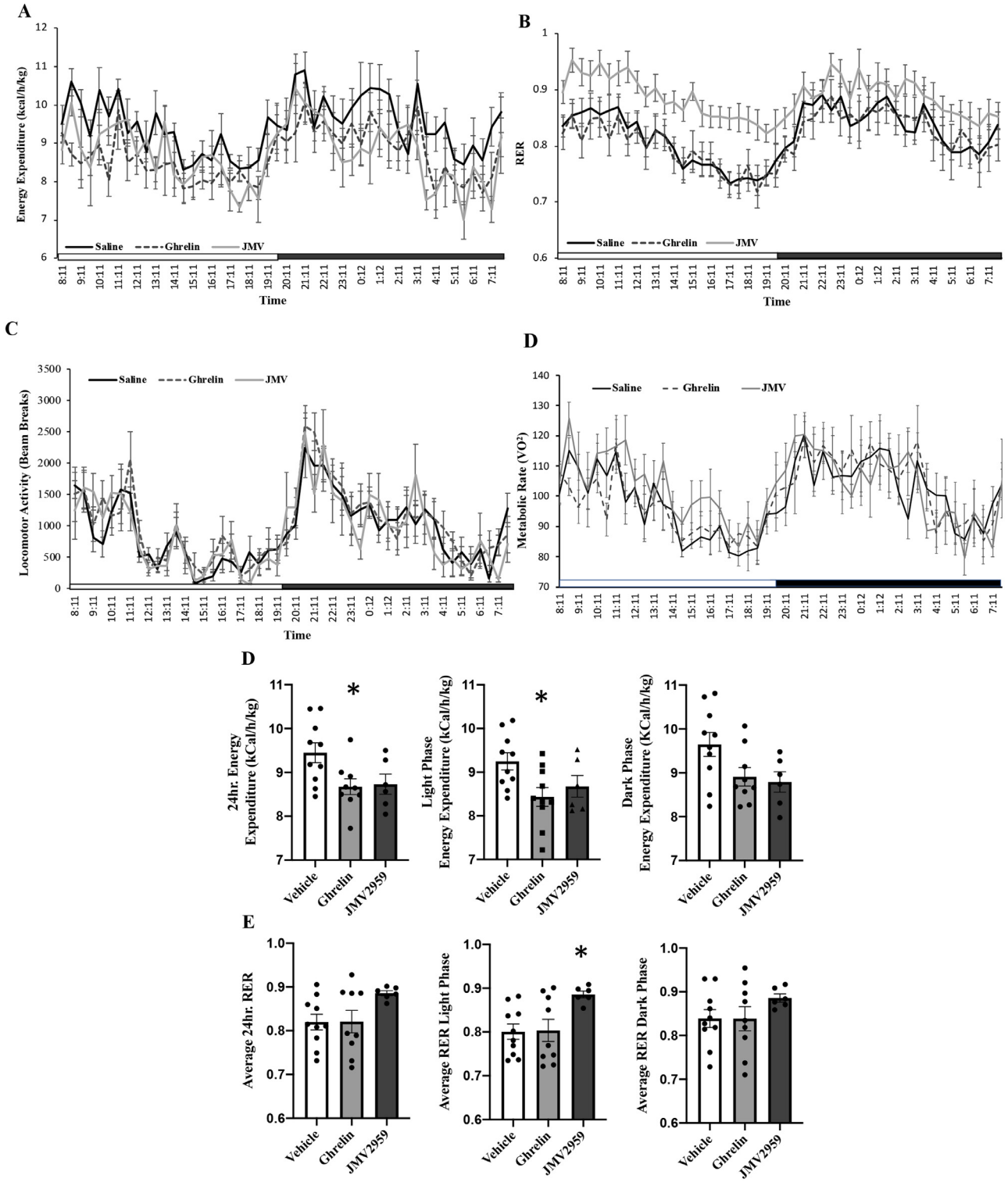

Fig. 3. Metabolic profile of mice infused with vehicle, ghrelin, and JMV2959 into the DMH. A shows energy expenditure (kcal/hr/kg), B shows RER and C shows locomotor activity in all mice throughout the last 24 h the mice were in the metabolic cages. White bar indicates the light phase, black bar indicates the dark phase. Data were analyzed as a total over the 24 h, and as total over the light and dark phases. As seen in D, ghrelin (but not JMV2959) treated mice showed a significant decrease in energy expenditure compared to vehicle treated mice, and this effect was significant only during the light phase of the light/dark cycle (See D). In contrast, JMV2959 significantly increased RER also in the light phase of the light/dark cycle. All data are expressed as mean +/− SEM. * $p < 0.05$.

in weight gain that is not accompanied by increased caloric intake [2]. Furthermore, GHSR1a and ghrelin KO mice, or double mutants for these genes are leaner in spite of consuming the same amount of food [27].

Chronic peripheral ghrelin treatment has been associated with fat accumulation through a bias towards carbohydrate use over fat, leaving adipose tissue to accumulate [2]. This is reflected in increased RER in ghrelin treated mice as shown by data obtained from indirect calorimetry measures [2]. An RER measure that is close to a value of 1.00 is presumed to be indicative of increased carbohydrate metabolism. In contrast, an RER value of 0.07 is thought to be indicative of increased

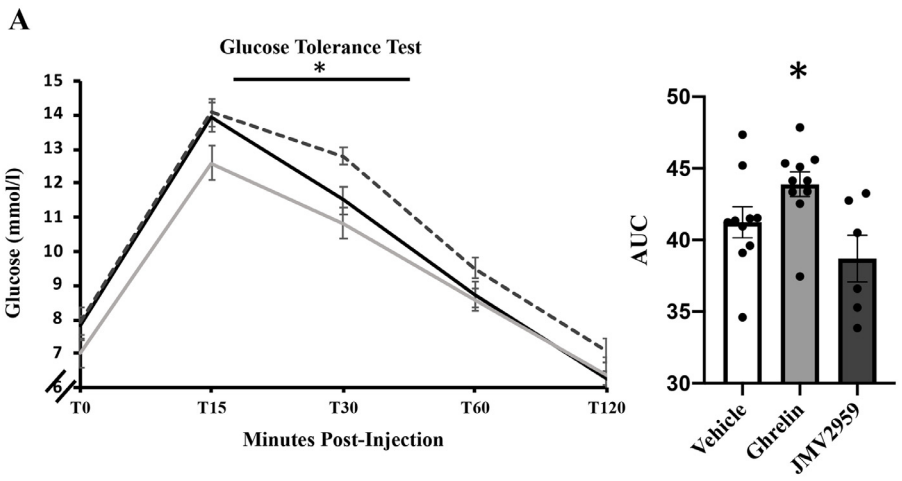

Fig. 4. Glucose tolerance (A) and area under the curve (AUC; B) in mice infused with vehicle, ghrelin or JMV2959 into the DMH. As seen in this figure, mice treated with ghrelin showed glucose responses that opposite to those of JMV2959, but neither were significant from vehicle treated mice. Ghrelin responses to the glucose load, however, were significantly higher than those of mice infused with JMV2959. All data are expressed as mean +/− SEM. * $p < 0.05$.

fat oxidation [2]. In our study, we did not observe changes in RER in response to ghrelin delivery into the DMH. We did, however, observe an increase in RER in response to JMV2959, suggesting that blocking the GHSR1a in the DMH affects substrate utilization in a way that mimics the global effects of ghrelin. While these results are at odds with what we expected, one could argue for the possibility that JMV2959 has effects that are not in line with those of pure antagonists, or that GHSR1a antagonists have some effects in the DMH that are similar to those agonists. Certainly, in vitro data suggests that while JMV2959 is a potent antagonist when in the presence of ghrelin, it has weak agonistic activity when applied in the absence of ghrelin [28]. Alternatively, in vivo treatment with the ghrelin receptor antagonists BIM28161 result in increased weight gain and activation of the DMH, results that would also support the notion that some GHSR1a antagonists have agonistic activity in the absence of ghrelin [29]. In this experiment, however, a potential for an agonistic action of JMV2959 can be discarded given that ghrelin in the DMH did not influence RER. It is perhaps more likely that JMV2959 is having an effect that is off target, and this effect needs to be further examined to better understand the nature of this compound. This increase in RER, however, does not translate into increased fat accumulation in JMV2959 treated mice.

Finally, our results show that unilateral intra-DMH infusions of ghrelin and JMV2959 have modest effects on fasting blood glucose concentrations when delivered into the DMH. While neither of these treatments produced significant differences on blood glucose concentrations compared to vehicle treated mice during the glucose tolerance test, the doses of ghrelin and JMV2959 chosen for the current study were sufficient to alter glucose responses to the glucose tolerance tests so that ghrelin treated mice had higher plasma glucose than mice treated with the GHSR1a antagonist JMV2959. This would support the idea that ghrelin can act on the DMH to influence glucose concentrations. Indeed, there is evidence supporting the idea the DMH is important for the regulation of glucose homeostasis [11, 30, 31]. Neurons within the DMH are responsive to changes in circulating glucose, generally being stimulated by increases in glucose concentrations [32]. These DMH neurons are also activated in glucoprivic states including after injections of 2-deoxyglucose (2-DG), a glucose analogue that cannot be metabolized by cells and that, at low doses, causes feeding by competing with L-glucose for entry into cells [33, 34]. Interestingly, lesions to the DMH decrease 2-DG induced feeding, supporting the idea that the DMH contributes to the mechanisms that regulate glucose concentrations, specifically by detecting drops in glucose to generate feeding [33]. In addition, ghrelin also plays an important role in the response to low levels of glucose, and this is reflected in the fact that mice with mutations to the ghrelin, GHSR1a, or the ghrelin-o-acyl transferase (GOAT; the enzyme that adds n-octanoic acid the ghrelin peptide, a modification required for ghrelin to bind to the GHSR1a) genes, have difficulties generating the glucogenic responses required to deal with prolonged fasting periods (24–48 h) [17, 35, 36]. These mice, however, also resist diet induced deficits in glucose regulation [27, 35]. Furthermore, acute and chronic peripheral ghrelin treatment leads to increases in glucose concentrations and these increases are not dependent on ghrelin induced growth hormone secretion [4, 35]. Our data, along with these findings, would suggest that ghrelin receptors in the DMH play a role in glucose homeostasis.

The phenotype of ghrelin sensitive cells in the DMH is not known, however the DMH contains cells that produce glutamate, GABA, and acetylcholine (ACh), and these neurons project to brain regions implicated in the regulation of food intake, body temperature, heart rate, thermogenesis, and sympathetic activation. For instance, a subset of neurons within the ventral portion of the DMH release GABA as a neurotransmitter and project to the hypothalamic paraventricular nucleus (PVN) to inhibit the release of corticotropin releasing hormone, a peptide associated with decreased food intake and increased energy expenditure [25, 37]. Another subset of cells located in the dorsal portion of the DMH release glutamate as a neurotransmitter, and these project to the dorsal median raphe nucleus where they stimulate sympathetic activity to increase heat production and cardiovascular responses [25]. Finally, cholinergic neurons in the DMH project to the ARC where they release ACh to inhibit the activity of POMC neurons and through this mechanism, increase food intake [16]. We do not know, however, if GHSR1a is distributed in all of these sets of neurons or if it is selectively expressed in a subset of these to regulate feeding and metabolism.

In all, we show evidence that chronic unilateral infusions of ghrelin into the DMH are sufficient to increase weight gain, adiposity, and caloric intake. While to our knowledge there are no previous experiments investigating acute or chronic ghrelin action in the DMH, our results are largely consistent with the effects of chronic peripheral or ICV ghrelin, which is known to promote food intake and body weight in rodents [2]. It is also understood that ghrelin can act on its central receptors to increase peripheral glucose levels [4], and in accordance with this, intra-DMH ghrelin decreases glucose clearance in the glucose tolerance test. Future studies should investigate the phenotype of the GHSR1a-positive cells within the DMH to obtain a better idea of the potential pathways by which ghrelin signaling affects energy balance in this region.

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