Physiology & Behavior 228 (2021) 113208

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/physbeh

Metabolic effects of ghrelin delivery into the hypothalamic ventral premammilary nucleus of male mice.

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ARTICLE INFO

Keywords:
Obesity
Ghrelin
Ghrelin receptors
Hypothalamus
Metabolism
Energy expenditure
Respiratory exchange ratio
Indirect calorimetry
Adiposity

ABSTRACT

Ghrelin is a 28 amino acid peptide hormone that targets the brain to promote feeding and adiposity. The ghrowth hormone secretagogue receptor 1a (GHSR1a) is expressed within many hypothalamic nuclei, including the ventral premammillary nucleus (PMV), but the role of GHSR1a signaling in this region is unknown. In order to investigate whether GHSR1a signaling within the PMV modulates energy balance, we implanted osmotic minipumps connected to cannulae that were implanted intracranially and aiming at the PMV. The cannulae delivered either saline or ghrelin (10 μg/day at a flow rate of 0.11μL/h for 28 days) into the PMV of adult male C57BLJ6 mice. We found that chronic infusion of ghrelin into the PMV increased weight gain, promoted the oxidation of carbohydrates as a fuel source and resulted in hyperglycemia, without affecting food intake, or body fat. This suggests that ghrelin signaling in the PMV contributes to the modulation of metabolic fuel utilization and glucose homeostasis.

1. Introduction

Energy homeostasis is maintained through mechanisms that include the integration of metabolic signals and hormones to modulate food intake and energy expenditure. While much progress has been made in understanding these mechanisms, the neural control of metabolism remains to be fully understood. The hypothalamic arcuate nucleus (ARC) is a key region regulating energy homeostasis [1]. The ARC, a bilateral nucleus located at the base of the mediobasal hypothalamus, contains neurons that secrete neurpopetide y (NPY) and the agouti related peptide (AGRP) both of which increase food intake and decrease energy expenditure [2, 3]. In addition, the ARC contains cells that secrete a-melanocyte-stimulating hormone (a-MSH), a peptide that curbs appetite and increases metabolic rate [4]. Because cells in the ARC contain numerous receptors for metabolic hormones, respond to changes in circulating metabolic signals like glucose and free fatty acids, and project to most other hypothalamic and numerous extra-hypothalamic regions, it is considered critical for energy homeostasis [1, 2]. Ghrelin, a hormone secreted by the stomach, binds to the growth hormone secretagogue receptor 1a (GHSR1a), a receptor that is expressed abundantly in NPY/AGRP neurons in the ARC [5-7]. Ghrelin stimulates appetite and decreases energy expenditure by acting on these neurons and indirectly by inhibiting neurons that produce a-MSH [7]. However, in addition to the ARC, other regions within the hypothalamus also express GHSR1a [8, 9].

A region that has received little attention with this regard is the ventral premammilary nucleus (PMV), a bilateral nucleus located in the posterior portion of the mediobasal hypothalamus, and one that is caudal to the ARC, and anterior to the superior mammillary nucleus [8, 9]. The PMV has been studied within the contex of the integration of nutritional signals to regulate reproductive function and behavior [10-14]. Indeed, the PMV has reciprocal projections with sexually dimorphic brain nuclei, such as the preoptic area (POA), medial amygdala (MeA), and lateral septal nucleus [15-19]. It also receives and sends signals to many feeding centres, including the arcuate nucleus of the hypothalamus (ARC), ventromedial hypothalamus (VMH), paraventrivular nucleus of the hypothalamus (PVN), and lateral hypothalamic area (LHA), among others [11, 19-21]. PMV neurons express the satiety neuropeptide CART, and receptors for cannabinoids (CB1R; Allen Mouse Brain Atlas; https://mouse.brain-map.org/experiment/show/283), and leptin [10, 16, 18, 22]. Notably, approximately half of PMV neurons express the leptin receptor and are activated by leptin administration [22-25], and its role in regulating reproduction within this region is well defined [11].

In addition to leptin receptors, neurons in the PMV also express ghrelin receptors in relatively high concentrations [8], yet the effects of ghrelin in this region have not been explored. In the current experiment we examined the effects of direct delivery of ghrelin onto the PMV on

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https://doi.org/10.1016/j.physbeh.2020.113208
Received 30 June 2020; Received in revised form 29 September 2020; Accepted 13 October 2020
Available online 14 October 2020

feeding and metabolism on male mice.

2. Method

2.1. Animals

All procedures were approved by the Carleton University Animal Care Committee and following the guidelines established by the Canadian Council on Animal Care (CCAC). Male mice (N = 20 C57BL/J6, 20–25 gs) were obtained from Charles Rivers farms (St. Constant, Quebec). Fig. 1A depicts the experimental timeline followed in this study. All mice were individually housed in clear plexiglass cages with a block of wood 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 06,414, Harlan; 5.2 kcal/g) was supplied between 8:30 am and 12:30 pm each day to determine if ghrelin would increase the intake of this diet at times in which mice do not consume food unless it is palatable. As shown in Fig. 1A, baseline measures of food intake and body weight were collected for 10 days to acclimate the animals to the daily handling and to the high fat diet. Baseline measures were used to create treatment groups made up with subjects that were matched body weight and food intake. Mice were assigned to one of two experimental drug treatment groups: vehicle (n = 10), or ghrelin (n = 10; 10 μg/day in isotonic saline; Peptides International). All mice underwent surgery to implant an intracranial cannula

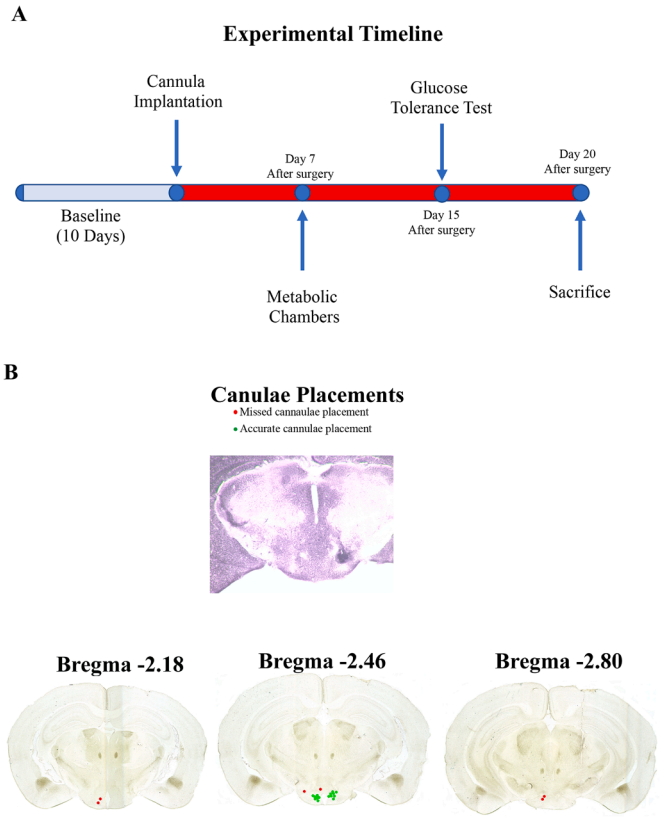

Fig. 1. Experimental timeline and cannulae placements for all experimental animals. (A) Depiction of experimental timeline from baseline to the end of the drug delivery period. (B) Coronal sections adapted from the Paxinos and Franklin Mouse Brain Atlas [23], depicting the PMV from anterior to posterior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

attached to an osmotic minipump, delivering drug or vehicle (Alzet Mini-Osmotic Pump – Model 1004; flow rate 0.11μL/h for 28 days). Surgical procedures were based on those described in previous work from our lab [26, 27]. In short, 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-gage stainless steel unilateral cannula (Alzet Brain Infusion Kit) coupled via a polyethylene catheter to a filled osmotic minipump was implanted into the PMV. Stereotactic coordinates relative to bregma were: AP 2.6 mm, ML 0.3 mm, and DV 5.4 mm using the Paxinos and Franklin Mouse Brain Atlas [28]. 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. Food intake (regular chow and HFD), and body weight were recorded daily until the end of the study (Day 21 post-surgery; See Fig. 1A for a timeline).

2.2. Metabolic chambers

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 hrs. The metabolic chambers were calibrated before the onset of the study and the flow of gasses was set at 0.25 L/minute. These boxes allowed us to measure oxygen consumption (VO_2), carbon dioxide production (VCO_2), food intake, and water intake every 30 min for 48 hrs. These measures were used to calculate the respiratory exchange ratio (RER; the ratio of the amount of VCO_2 produced to the amount of O_2 consumed), an indirect measure of substrate utilization (REF). We also calculated energy expenditure (kcal/h/kg) based on the caloric value (CV) of RER, oxygen consumption, and body weight of the animal: $\frac{CV(of\ RER)*VO2\left(\frac{ml}{h}\right)}{body\ weight(kg)}$. Metabolic chambers allowed for the recording of locomotor activity as a function of infrared beam breaks. Here we report total locomotor activity (all beam breaks in the x, y and z axis). While mice were placed in the chambers and data was collected for 48 hrs, we only used the data from the last 24 hrs, as the first 24 hrs are typically used to acclimate the mice to the setup. After 48 hrs, mice were returned to their home cages.

2.3. Glucose tolerance test and body composition analysis

On day 15 after cannula implantation, mice underwent a glucose tolerance test to determine the rate of glucose clearance from blood. On the evening prior to the test (Day 14 after surgery), 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 Countour Next blood glucose test strip was inserted into a Countour 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 2 g / kg of body mass. Blood was collected 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.

Mice were sacrificed twenty days after the surgery by rapid

decapitation and brains were postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer at a pH of 7.3. Carcasses were frozen at −20° until analyzed for body composition analysis using an EchoMRI Body Composition Analyzer EF-020 (Houston, Texas, www.echomri.com). Carcasses were thawed prior to being scanned.

2.4. Cannula placements

Brains were extracted and stored in 4% PFA in PB for at least 72 hrs 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 0.1% sodium azide), in order to cryoprotect the brains before slicing. Once the brains sank to the bottom of the vial, they were sliced at 40um 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 data were converted into calories and analyzed using repeated measures ANOVAS with days after surgery as the within group factor and treatment as the between group factor. Similar ANOVAS were conducted for cumulative weight gain and for the measures from the metabolic cages over time. Repeated measures ANOVAS for daily caloric intake and cumulative weight gain used data from the first 14 days of treatment only, as mice were fasted for the glucose tolerance test after that time. In addition, independent t-tests were used to compare total cumulative caloric intake and weight gain throughout the experiment. Similar tetss were conducted on other caloric intake measures (chow caloric intake, and high fat diet caloric intake), body composition (total fat, total lean, ratio of fat mass to body weight and ratio of lean mass to body weight, RER, EE, locomotor activity). Glucose levels across the test were analyzed by repeated measures ANOVA, with time as the within-subjects factor, and drug treatment as the between-subjects factor. Area under the curve (glucose tolerance test) were all analyzed as total using an independent samples *t*-test. Where appropriate, Tukey's post-hoc comparisons were used.

3. Results

A total of 20 mice were used in the study. Of these, two animals did not recover from surgery and were euthanized. The baseline data from these mice were removed from all analyses. Following cannula placement inspection, four additional animals were removed due to incorrect cannula placements (see Fig. 1b for placement location). Thus, a total of 14 animals were included in the final analyses, with the following group numbers: saline ($n = 6$), ghrelin ($n = 8$).

Fig. 2 shows the effects of vehicle or ghrelin on total caloric intake, and caloric intake of chow or high fat diet. Repeated measures ANOVAs on these measures showed no significant effects of treatment nor interaction effects, suggesting that ghrelin infusion into the PMV did not influence the intake of chow, high fat diet, or the overall caloric intake across the first two weeks of the study. There seemed to be a small effect of ghrelin on caloric intake from chow during the first three days after the surgery, but this increase was not statistically significant as determined by the lack of a treatment or an interaction effect ($p. > 0.5$). When examining the total coloric intake, or the individual caloric intake of chow or the high fat diet, it appeared that ghrelin produced a small increase in the intake of calories from chow, and in the overall total caloric intake throughout the experiment, but this effect was not statistically significant ($p = 0.07$ for total caloric intake; $p = 0.20$ for total calories from chow; $p = 0.76$ for calories from high fat diet; See Fig. 2A, B,C).

Seven days after the onset of ghrelin infusions, animals were moved to metabolic chambers to determine if ghrelin delivery into the PMV

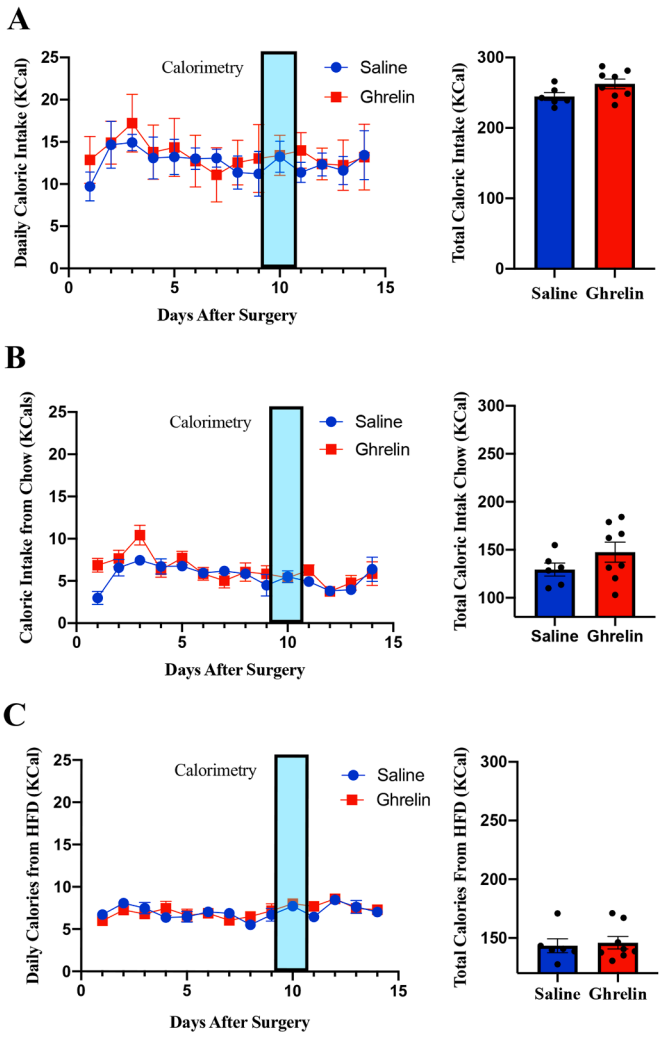

Fig. 2. Average overall caloric intake (A), calories from chow (B) and calories from fat (C). Shaded area represents the days in which mice were tested in the metabolic chambers. On the left graphs depict the intake over the first 14 days of the experiment, and on the right the bar graphs depict the total calories consumed throughout the study. As shown in this figure, ghrelin infused into the PMV did not produced any substantial increase in caloric intake ($P. > 0.05$). All data are expressed as mean +/- SEM.

affected metabolism. As shown in Fig. 3, a repeated measures ANOVA determined that chronic infusion of ghrelin over the first week of treatment led to a significant increase in the respiratory exchange ratio determined by a significant treatment main effect ($F(1,12)=11.01$, $p.< 0.05$). The average respiratory exchange ratio was significantly higher in ghrelin treated mice than in vehicle tread mice during the light and the dark period across the testing day (t(12)= 2.64 Lights On; t(12)= 3.005 Lights Off; $p. < 0.05$; see Fig. 3A). In contrast to RER, energy expenditure was not affected by ghrelin infusions into the PMV, and measures of energy expenditure were virtually identical in ghrelin and vehicle treated mice over the testing period ($p. > 0.05$; see Fig. 3B). Another source of energy expenditure is locomotor activity, but similar to energy expenditure measured using indirect calorimetry, locomotor activity (as measured by the number of infrared beam breaks over the testing period) was not different in ghrelin and vehicle treated mice($p. > 0.05$; see Fig. 3C).

The changes in respiratory exchange ratio observed after the first week of treatment would be predictive of weight gain and fat deposition. Indeed, a seen in Fig. 4A, ghrelin infusions into the PMV appeared to increase cumulative weight gain in ghrelin treated mice, particularly

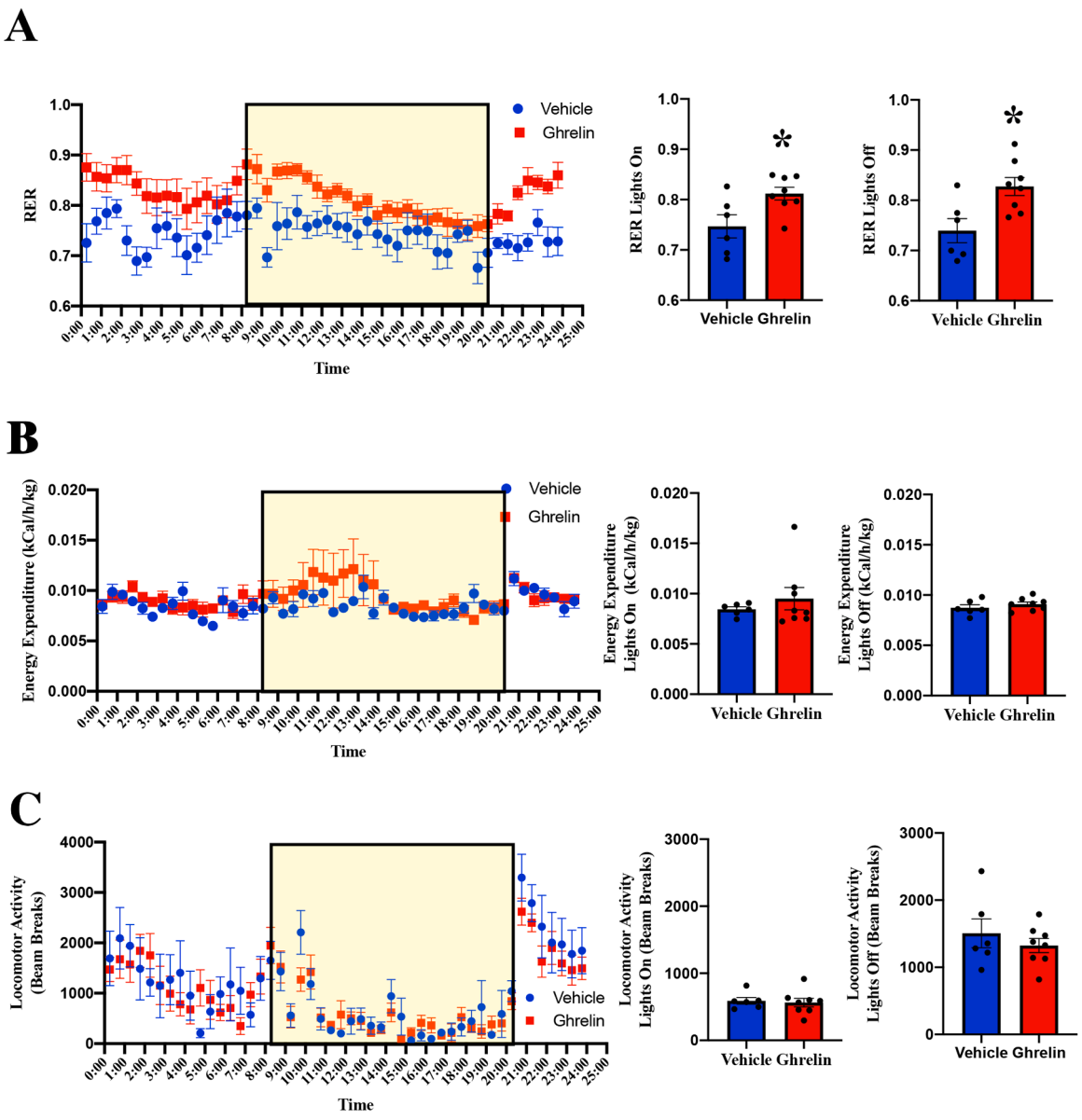

Fig. 3. Metabolic profile of mice infused with vehicle or ghrelin into the PMV. Fig. 3A shows RER across the 24 hour period; shaded area indicates the light phase of the 24hr cycle. Bar graphs indicate the average RER in the light and dark phase of the 24 hour light dark cycle. As shown in this panel, ghrelin infusions into the PMV resulted in a significant increase in RER, and this increase was evident in both the light and dark phases of the cycle ($p. < 0.05$)Fig. 3B and 3C show energy expenditure (kcal/hr/kg) and locomotor activity (infrared beam breaks) across the 24 hour period. No significant difference between saline and ghrelin in energy expenditure or locomotor activity were observed in these measures ($p. > 0.05$). during either the dark or light phases of the light-dark cycle. All data are expressed as mean +/- SEM. * $p < 0.05$.

over the second week of treatment. A repeated measures ANOVA however, determined that this increase was not statistically significant (interaction effect, $F(13,156) = 1.66$, $p. = 0.07$). When we analyzed the total weight gain of ghrelin versus vehicle treated mice over the treatment period (body weight on day19-body weight on day of surgery) we observed that mice infused with ghrelin into the PMV gained significantly more weight than mice infused with saline ($t(12) = 2.216$, $p. < 0.05$; see Fig. 4A). This increase in weight gain however, was not accompanied by an increase in adiposity as measured by body composition analyses using EchoMRI. As shown in Fig. 4B, there were no significant differences in the proportion of fat mass, lean mass or water content between the treatment groups($p. > 0.05$). Interestingly, while chronic infusions of ghrelin into the PMV did not result in increased adiposity, they did alter the response to a glucose load as determined by the glucose tolerance test. As shown in Fig. 4C, a repeated measures ANOVA detected an overall ghrelin effect on plasma glucose concentrations after a glucose challenge (main effect of treatment, $F(1,12) = 14.22$, $p. < 0.05$). The effect of ghrelin delivery on glucose regulation was also reflected by a significant increase in the area under the curve of ghrelin infused mice compared to vehicle infused mice ($t(12) = 3.507$, $p. < 0.05$; see Fig. 3C).

4. Discussion

In this experiment we examined the effects of chronic ghrelin delivery into the PMV on food intake and energy metabolism. Our data shows that chronic ghrelin delivery into this discrete hypothalamic region were effective in producing a number of metabolic changes that resulted in increased weight gain and altered glucose regulation. Notably, these changes were observed in the absence of significant increases in caloric intake or fat deposition.

Acute or chronic delivery of ghrelin into different hypothalamic

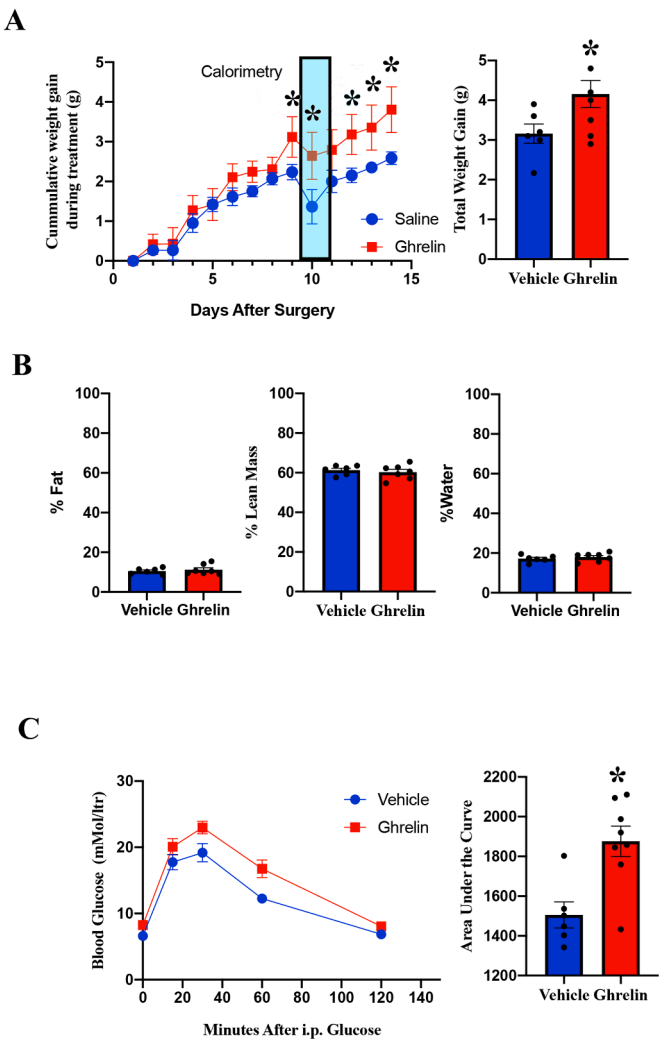

Fig. 4. Cummulative weight gain (Panel A), body composition (Panel B) and glucose tolerance (Panel C) in mice chronically infused with vehicle or ghrelin into the PMV. As seen in this figure, mice treated with ghrelin showed an increase in weight gain in the second week of treatment and this increase was significant by the end of the experiment ($p. < 0.05$; Panel A). This increase in weight produced by ghrelin, however, was not followed by increases in adiposity, lean mass or water ($p.> 0.05$; Panel B). Glucose tolerance tests demonstrated that ghrelin infusions into the PMV resulted in significantly higher glucose concentrations after a glucose challenge and a higher area under the curve compared to saline infused mice. All data are expressed as mean +/- SEM. * $p < 0.05$.

nuclei has consistently been associated with increased caloric intake [27, 29-32]. In the current study we observed a small increase in overall caloric intake in ghrelin infused mice, but this effect did not attain statistical significance. This is interesting considering that the PMV rests rostro-caudally between the DMH and the VTA, two regions that express GHSR and that produce feeding responses when infused chronically with ghrelin [27, 33, 34]. Furthermore, chronic ghrelin delivery into the PMV was not effective in increasing the intake of a high fat diet, a diet that is generally preferred by C57BL/J6 mice [35]. We also observed a small increase in calories sourced from the chow diet, but this effect was only observed in the days following the surgical procedure, and were not statistically significant, leading us to conclude that ghrelin delivery into the PMV has minimal effects on caloric intake or diet preference. Acyl-ghrelin is rapidly degraded in circulation by esterases found in circulation [36-39], and as such, the stability of ghrelin in a minipump for prolonged periods of time could be questioned. Osmotic minipumps are, however, sterile and likely devoid of esterase activity. As such, it is unlikely that synthetic ghrelin freshly reconstituted and loaded into the minipumps would degrade as it would in circulating blood. This contention is supported by previous work showing that chronic peripheral and central ghrelin treatment using osmotic minipumps is effective in increasing food intake compared to controls without evidence of hormone degradation after periods ranging between 7 and 21 days [5, 27, 34, 40-42]. Thus, it is unlikely that the lack of food intake effect was due to degradation of the peptide, although we acknowledge that this is an issue that requires further examination.

While ghrelin infusions into the PMV did not increase caloric intake substantially, ghrelin delivery into the PMV did produce an increase in RER in mice tested after the first week of infusions. The effect of ghrelin infusions into the PMV on RER were evident both in the light and the dark phases of the testing day. This is in line with the effects of ghrelin on RER reported by previous experiments where ghrelin is delivered peripherally, into the cerebral ventricles, or into specific hypothalamic regions [7, 29, 43]. These data support not only the idea that ghrelin has site specific metabolic effects in the PMV, but also that ghrelin remained patent inside the minipump at least during the first 7–10 days of infusion. This suggests that ghrelin can influence the preferential use of carbohydrates as a source of fuel, sparing fat.

Given the effects of ghrelin on RER after one week of infusion we expected that ghrelin treated mice would gain weight and would also show increased fat deposition. In line with this, we observed a significant increase in weight gain in animals treated with ghrelin infused into the PMV. While the weight gain was not fully evident two weeks into the infusion treatment, mice chronically treated with ghrelin into the PMV gained more weight than vehicle treated mice by the end of the testing period. Unexpectedly, when body composition analyses were conducted, we did not observe changes in the proportion of fat mass to body weight in ghrelin treated mice. We also failed to detect changes in lean mass or in water content in ghrelin treated mice. The paradoxical change in weight in the absence of increased adiposity could be explained by a change in the distribution of fat across different fat pads, an effect that we would not be able to detect with the EchoMRI instrument. Alternatively, it is possible that weight gain produced by ghrelin is caused by an overall change in body size. Indeed, electrical stimulation to regions that include the PMV as targeted in this study result in marked increases in the release of growth hormone [44], whereas lesions to this region result in growth retardation [45]. Also neurons within the PMV express growth hormone-releasing hormone, and these project to the median eminence [46].This would suggest that ghrelin acting on this region could increase the growth rate of mice without necessarily affecting body composition.

Two weeks after chronic ghrelin or saline delivery, mice were tested on the glucose tolerance test to determine differences in glucose regulation produced by this treatment. Results from this test showed that ghrelin delivery into the PMV resulted in mice with overall higher glucose concentrations and a higher area under the curve in the glucose tolerance test, suggesting that ghrelin delivery into the PMV might cause a mild state of hyperglycemia. The mechanisms underlying these effects are not yet known. One possibility is that ghrelin delivery into the PMV produces endocrine changes that result in increased glucose release. As mentioned above, the PMV is associated with the release of GH [44, 45]. It is therefore possible that ghrelin stimulates growth hormone release via actions onto the PMV, and these increases in growth hormone may ultimately lead to increased glucose production and utilization as substrate as shown by increased RER in our ghrelin infused mice. Alternatively, given that PMV stimulation also results in acute elevation of corticosterone (CORT) secretion in anaesthetized rats [47], and that central ghrelin delivery increases the release of adrenocorticotropin hormone (ACTH) and CORT [48, 49], increased glucose levels seen in ghrelin treated animals in our current experiment could therefore be related to increases in CORT. Ultimately, these high glucose concentrations may also explain why ghrelin treated mice also showed elevated RER, as increased glucose concentrations may bias metabolic use of

carbohydrates over fat.

In the current study we used unilateral delivery of ghrelin or saline given that it is difficult to equip mice with two minipumps, and also difficult to implant two cannula that are targeting bilateral hypothalamic nuclei that are close to the midline in mice. It is therefore possible that we did not observe a significant caloric intake effect because that may have required bilateral delivery of ghrelin. However, unilateral delivery of ghrelin into regions adjacent to the PMV result in increased food intake. For example, chronic unilateral ghrelin delivery into the DMH a region just anterior to the PMV, or the VTA which is just posterior to the PMV, result in increased food intake [27, 29, 32, 33]. Similarly, unilateral ghrelin infusions into the lateral hypothalamus, ARC, VMH and PVN also result in increased food intake [29, 30, 43]. These data rather indicate that the PMV does not play a significant role in the orexigenic responses that are commonly associated with ghrelin.

Drug delivery using minipumps is a convenient way of elevating tonic drug concentrations in specific target brain regions without excessive handling of the animals. One drawback is that continuous delivery can cause spillover of the drug into adjacent regions, a potential confound when looking at effects within discrete hypothalamic nuclei. We, however, do not believe this is the case. If ghrelin spillover was producing the effects of ghrelin on weight gain or RER observed in this study, we would have observed an effect on food intake, a hallmark in the effects of ghrelin within other hypothalamic areas. Also, while effects of ghrelin on RER have been reported with infusions into the PVN, ARC and VMH, that is not the case with infusions into the DMH, a region just rostral and dorsal to the PMV [27, 29, 30, 43]. Thus, it is more likely that the effects observed are specific to GHSR stimulation in cells within this region than stimulation of ghrelin sensitive cells in adjacent hypothalamic nuclei.

The dose chosen for this study is one that we have used in the past successfully to demonstrate the effects of ghrelin on food intake and energy balance within the DMH [27]. At the outset, the dose of 10 μg/day may seem excessive, but this dose was delivered throughout the day continuously a very low rate. In reality the mice were receiving around 0.42 μg every hour and 0.007 μg per minute. As such, this way of delivering ghrelin causes a tonic increase in ghrelin within the targeted region, and the amount of vehicle used to deliver the drug was unlikely to cause damage to the PMV. We are therefore confident that the effects observed are due to biological consequences of increased tonic ghrelin than due to a pharmacological effect caused by an acute dose of ghrelin that is 10–100 times larger. Finally, while we cannot discard the possibility that ghrelin delivered in this manner is transported from the brain to the periphery, it is doubtful that the effects observed would be due to peripheral effects given how low the dose is, and how fast ghrelin is degraded by esterases found in circulation [37-39].

The PMV is a sexually dimorphic region and in males it appears to be important for social investigation and is a nucleus that is responsive to the presence of male intruders in both male and female mice [50, 51]. Optogenetic activation of the PMV induces social investigation and aggression in male mice, suggesting that in males, this region is important for behaviors associated with sexual competition [52]. In females, the PMV has been implicated in the response to metabolic hormones like leptin to modulate reproductive function [11, 14]. Leptin signaling in the PMV is a permissive signal for the onset of puberty in female mice and rats [11, 14]. Mice that do not have leptin receptors are infertile, but selective rescue of leptin receptors in the PMV restores fertility in these mice [53]. It is not clear if GHSR expressing cells in the PMV are also sensitive to leptin, but there is evidence that ghrelin can regulate the reproductive function in female rodents [54-57], so it is likely that the PMV monitors ghrelin levels as an index of food availability to modulate reproduction, but research linking the PMV to the effects of ghrelin on reproduction is still needed. Indeed, the metabolic effects of ghrelin are mitigated by estradiol and enhanced when ovaries are removed in mice [58]. Studies in female mice are needed to better understand this sex specific relationship between feeding and reproduction.

In all, our results support the idea that the PMV is responsive to ghrelin and GHSR stimulation in this region can increase weight gain and carbohydrate utilization and the release of blood glucose. While GHSR in this region might not be key to the adipogenic and orexigenic effects of ghrelin, GHSR in this region does integrate some metabolic effects of ghrelin which could be integrated to modulate other responses produced by the PMV including reproductive and social behaviors. Ultimately, understanding how chronically elevated levels of ghrelin influence metabolic processes in different hypothalamic brain regions may provide clues for better understanding of the role of ghrelin on energy balance under normal condition and in conditions that are associated with clinical metabolic dysregulation like Prader-Willi syndrome, a syndrome associated with hyperghrelinemia [59-61].

Acknowledgements

This work was supported by a grant from the Canadian Institutes for Health Research awarded to AA. This work was also supported by a Queen Elizabeth II Graduate Scholarship awarded to LH.

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