

**The Role of Growth Hormone Secretagogue Receptor (GHSR) Signaling in the
Dorsomedial Hypothalamus (DMH) and Ventral Premammillary Nucleus (PMV) In
Energy Homeostasis in Mice**

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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 hypothalamic nuclei, including the DMH and PMV, but the role of GHSR1a signaling in these regions is unknown. In order to investigate whether GHSR1a signaling within these regions modulates energy balance, we conducted two experiments. In study 1, we attached a minipump filled with saline, ghrelin, or a GHSR1a antagonist to a cannula aimed at the DMH in adult male C57BLJ6 mice and assessed their metabolic profile. In study 2, we employed similar drug treatments as in study 1, but aimed the cannula at the PMV. We found that chronic stimulation of the GHSR1a in the DMH leads to an increase in body weight, primarily in the form of adipose tissue, without affecting caloric intake. The increase in body fat is accompanied by and may be due to a decrease in energy expenditure, which is not associated with a decrease in locomotor activity. Further, chronic stimulation of the GHSR1a in the PMV as well as the DMH slows glucose clearance. However, infusion of ghrelin into the PMV promotes the oxidation of carbohydrates as a fuel source, without affecting food intake, body weight, or body fat. This suggests that GHSR signaling has distinct roles in the DMH and PMV in maintaining energy homeostasis.

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List of Abbreviations

α -MSH, alpha-melanocyte-stimulating hormone; **AAV**, adeno-associated virus; **AgRP**, agouti-related peptide; **AMP**, adenosine monophosphate; **AMPK**, adenosine monophosphate activated protein kinase; **ANOVA**, analysis of variance; **ARC**, arcuate nucleus; **BAT**, brown adipose tissue; **cAMP**, cyclic adenosine monophosphate; **CART**, cocaine amphetamine regulated transcript; **CB-1R**, cannabinoid receptor 1; **CB-2R**, cannabinoid receptor 2; **CCK**, cholecystokinin, **CCK1R**, cholecystokinin 1 receptor; **CNS**, central nervous system; **CPP**, conditioned place preference; **CRF**, corticotrophin-releasing factor; **CSF**, cerebrospinal fluid; **DMH**, dorsomedial nucleus; **FEO**, food-entrainable oscillator; **GABA**, gamma-aminobutyric acid; **GAL**, galanin; **GLP-2**, glucagon-like peptide 2; **GPCR**, G-protein coupled receptors; **GH**, growth hormone; **GHSR**, growth hormone secretagogue receptor; **GHSR KO**, growth hormone secretagogue receptor knockout; **GOAT**, ghrelin O-acyltransferase; **HFD**, high fat diet; **IP**, intraperitoneal; **IP₃**, inositol (1,4,5) triphosphate; **LepR**, leptin receptor; **LH**, lateral hypothalamus; **LHA**, lateral hypothalamic area; **LS**, lateral septal nucleus; **MC3R**, melanocortin-3 receptor; **MC4R**, melanocortin-4 receptor; **MeA**, medial amygdala, **mPOA**, medial preoptic area; **mRNA**, messenger ribonucleic acid; **NA**, noradrenaline; **NPY**, neuropeptide Y; **NST**, non-shivering thermogenesis; **NTS**, nucleus of the tractus solitaries; **OLETF**, Otsuka Long-Evans Tokushima Fatty; **PFA**, paraformaldehyde; **PLC**, phospholipase C; **PMV**, ventral premammillary nucleus; **PNS**, peripheral nervous system; **POA**, preoptic area; **POMC**, pro-opiomelanocortin; **PVN**, paraventricular nucleus; **RFS**, restricted feeding schedule; **RMR**, rostral medullary raphe; **rRP**, rostral raphe pallidus; **SCN**, suprachiasmatic nucleus; **SNS**, sympathetic nervous system; **SPN**, sympathetic premotor neuron; **sPVZ**, subparaventricular zone; **TRH**, thyrotropin releasing hormone; **UCP1**, uncoupling protein 1; **UCP2**, uncoupling protein 2; **UCP3**, uncoupling protein 3; **VMH**, ventromedial nucleus of the hypothalamus; **VP**, ventral pallidum; **VTA**, ventral tegmental area; **WAT**, white adipose tissue; **WT**, wild-type

Table of Contents

Abstract	2
Acknowledgements	3
List of Abbreviations	4
Table of Contents	5
List of Tables and Figures	6
Introduction	8
Ghrelin and the regulation of energy balance	9
Peripheral ghrelin actions in energy balance	12
Central actions of ghrelin.....	14
The DMH.....	17
DMH control of food intake	19
DMH control of thermogenesis	20
DMH control of circadian rhythms.....	23
Ghrelin in the DMH.....	25
The ventral premammillary nucleus	26
Aims and Hypotheses	29
Methods	31
Subjects.....	31
Timeline	31
Surgical Procedure	31
Metabolic Chambers	32
Glucose Tolerance Test.....	33
Cannula Placements.....	34
Statistical Analysis.....	34
Experiment 1 Groups and Drugs.....	36
Experiment 2 Groups and Drugs.....	36
Experiment 3 Groups and Drugs.....	37
Results	37
Experiment 1 Determination of JMV2959 Dose	37
Experiment 2 Effects of Manipulating GHSR signaling within the DMH	39
Experiment 3 Effects of Manipulating GHSR signaling within the PMV	57
Discussion	66
Appendix	75
References	76

List of Tables and Figures

Introduction

Figure A: Depiction of projections to and from the DMH..... 18

Figure B: Depiction of projections to and from the PMV 27

Methods

Figure C: Depiction of experimental timeline and experimental days of each phase 31

Results

Study 1

Table 1: Summary of study 1 results 38

Study 2

Figure D & E: Images of representative cannula placements within the DMH 39

Figure F: Summary of cannula placements for experiment 2 39

Figure 1A & 1B: Overall and daily body weight change 40

Figure 2A & 2B: Overall and daily chow intake change 41

Figure 3: Daily high fat diet intake change 42

Figure 4A & 4B: Overall and daily total caloric intake change 43

Figure 5A & 5B: Overall and daily body weight change during phase 1 44

Figure 6: Daily chow intake change during phase 1 44

Figure 7: Daily total caloric intake change during phase 1 45

Figure 8A & 8B: Overall and daily body weight change during phase 2 46

Figure 9: Daily chow intake change during phase 2 47

Figure 10: Daily total caloric intake change during phase 2 48

Figure 11: Daily body weight change during phase 3 49

Figure 12: Daily total caloric intake change during phase 3 49

Figure 13: RER values at 30 minute intervals across a 24 hr period 50

Figure 14A & 14B: Mean lights on and lights off RER values 51

Figure 15: Energy expenditure values at 30 minute intervals across a 24 hr period 52

Figure 16A & 16B: Mean lights on and lights off energy expenditure values 52

Figure 17A & 17B: Glucose clearance curve and area under the curve 54

Figure 18A & 18B: Ratio of fat mass to body weight and ratio of lean mass to fat mass 55

Figure 19: Anticipatory locomotor activity 56

Study 3

Figure G: Summary of cannula placements for experiment 3 57

<i>Figure 20: Overall daily body weight change</i>	58
<i>Figure 21: Overall daily chow intake change</i>	59
<i>Figure 22: Overall daily high fat diet intake change</i>	60
<i>Figure 23: Overall total caloric intake change</i>	61
<i>Figure 24: RER values at 30 minute intervals across a 24 hr period</i>	62
<i>Figure 25A & 25B: Mean lights on and lights off RER values</i>	62
<i>Figure 26A & 26B: Glucose clearance curve and area under the curve</i>	63
<i>Figure 27A & 27B: Ratio of fat mass to body weight and ratio of lean mass to fat mass</i>	64
<i>Figure 28: Anticipatory locomotor activity</i>	65

Appendix

<i>Table 2: Summary of study 2 high fat diet and chow intake change during phases 2 & 3</i>	75
<i>Table 3: Summary of study 2 VCO₂, VO₂, and locomotor activity</i>	75
<i>Table 4: Summary of study 2 ANCOVAs for RER and energy expenditure</i>	75
<i>Table 5: Summary of study 3 VCO₂, VO₂, locomotor activity, and energy expenditure</i>	75

Introduction

Obesity is considered to be the leading cause of preventable death, and results in increased risk and severity of cardiovascular disease, type two diabetes, and cancer, among other comorbid pathologies. Obesity results from a dysregulation of energy balance, thus research furthering the understanding of systems controlling energy balance is useful in developing long term solutions to the obesity epidemic. Energy homeostasis is maintained by regulating food intake and energy expenditure, processes which are controlled through many converging mechanisms. While much progress has been made in understanding these mechanisms, the neural control of metabolism remains to be fully understood. It is, however, known that the hypothalamus plays a major role in this control, and involves the coordinated actions of peripheral systems including the GI tract, liver, muscle, pancreas, white adipose tissue (WAT) and brown adipose tissue (BAT) (Munzberg et al., 2015). The central integrative circuits of the neural control of metabolism are sensitive to the actions of many circulating hormones that signal alterations in energy balance. One such hormone is ghrelin, a hormone produced by the stomach, which stimulates feeding behaviour and decreases fat utilization, ultimately increasing fat deposition (Tschop et al., 2000). 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 (Nakazato et al., 2001; Tsubone et al., 2005; Tschop et al., 2000; Broglio et al., 2001; Cui et al., 2008). The receptor for ghrelin, the growth hormone secretagogue receptor (GHSR), is expressed in many peripheral and central tissues, and in particular is expressed in abundance within the hypothalamus (Zigman et al., 2006; Kojima et al., 1999; Cummings, 2006; Depoortere, 2009). Ghrelin's orexigenic effects are largely accomplished through its actions in various nuclei of the hypothalamus, including the

paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and arcuate nucleus. However, the GHSR is also expressed within the dorsomedial nucleus of the hypothalamus (DMH) and the ventral premammillary nucleus (PMV), both of which are accessible to ghrelin via the cerebrospinal fluid (Cabral, Fernandez, and Perello, 2013), but ghrelin's actions within these regions are unknown.

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 (Gooley, Schomer, and Saper, 2006; Chou et al., 2015; Bellinger and Bernardis, 2002; Cao and Morrison, 2006; Chao et al., 2011; Jeong et al., 2015). Further, lesions of the DMH lead to diminished food and water intake, as well as reductions in body weight and growth (Bellinger and Bernardis, 2002). While the DMH is accessible to ghrelin via the CSF and expresses the GHSR, a greater expression of the GHSR is found within the PMV, an area of the hypothalamus located just posterior to the DMH (Zigman et al., 2006). The PMV is responsive to reproductive, metabolic, and social signals, including gonadal hormones, leptin and ghrelin, and odorants (Leshan and Pfaff, 2014), and hence is thought to be an integrative centre for peripheral signals. The overall goal of the studies described in this thesis is to investigate the metabolic effects of altering GHSR1a signaling within the DMH and PMV.

Ghrelin and the Regulation of Energy Balance

Ghrelin is a 28 amino acid orexigenic peptide hormone that plays an important role in energy metabolism by regulating food intake, body weight, and glucose homeostasis (Tschop et al., 2000). Ghrelin is secreted primarily by endocrine gastric X/A-like cells of the oxyntic

mucosa lining of the stomach (Kojima et al., 1999), but is also produced by other tissues including but not limited to the upper intestines, liver, pancreas, and pituitary (Date et al., 2000; Gnanapavan et al., 2002). The ghrelin gene codes for a 117 amino acid prepropeptide (preproghrelin), that is cleaved into proghrelin and obestatin (Gourcerol et al., 2007). Proghrelin becomes ghrelin upon the loss of its signal peptide, before being released into the circulation (Kojima et al., 1999). Ghrelin was originally discovered because of its ability to stimulate GH release from the pituitary (Kojima et al., 1999), and is the only known peripheral peptide that stimulates food intake. In contrast to leptin and insulin, ghrelin is secreted into the circulation in times of negative energy balance, such as when the stomach is empty and prior to scheduled meals (Cummings et al., 2001; Cummings et al., 2004). In order to bind to its receptor, ghrelin requires the addition of a fatty acyl sidechain onto the serine-3 residue, a post-translational modification that is catalyzed by the enzyme ghrelin O-acyltransferase (Gutierrez et al., 2008; Yang et al., 2008). Only about 10% of total ghrelin in the circulation is acyl-ghrelin (the active form of ghrelin), while the other 90% remains desacyl-ghrelin (Patterson et al., 2005; Yoshimoto et al., 2002). Although it cannot bind to the GHSR-1a and was originally believed to be inactive and a by-product of acyl-ghrelin, desacyl-ghrelin is now also thought to be important for energy balance in rodents (Thompson et al., 2004; Toshinai et al., 2006), including stimulation of food intake in a GHSR-independent manner (Toshinai et al., 2006).

The *GHSR1* gene, which encodes for the full GHSR1a that is responsive to ghrelin binding, also encodes for a truncated isoform (GHSR1b) that does not bind ghrelin and whose signaling activity is not well understood (Gnanapavan et al., 2002; Howard et al., 1996). Although the GHSR1b does not bind ghrelin, it is expressed widely, and has been shown to dimerize with the GHSR1a and reduce its cell surface expression, suggesting it is a negative

regulator of ghrelin signaling (Chow et al., 2012; Leung et al., 2007). The GHSR1a has a high degree of constitutive activity, about 50% of its maximal activity (Holst et al., 2004), and is thus thought to be physiologically important, however the degree to which constitutive activity contributes to the actions of the ghrelin system in specific biological functions is not known. To further complicate the understanding of the ghrelin system, there are multiple intracellular signaling pathways that are activated upon the binding of ghrelin to the GHSR1a that may each differentially contribute to ghrelin's wide array of functions.

The GHSR1a is a G-protein coupled receptor (Yin, Lee, and Zhang, 2014), and ghrelin binding induces activation of one of four G-proteins: G_q which stimulates PLC production, G_i which inhibits cAMP production, G_o which governs the PI3K/mTOR pathway, and G_{13} which induces MAPK activity (M'Kadmi et al., 2015). Of these signaling cascades, the best established and characterized is the phospholipase C (PLC)/inositol (1,4,5) triphosphate (IP_3) mediated increase in $[Ca^{2+}]$ that occurs via G_q activation (Chen, 2000). The PLC/ IP_3 pathway was the first to be identified as occurring as a result of constitutive activity of the GHSR1a (Petersenn, 2002; Adams et al., 1995; Lei et al., 1995; Chen, Wu, and Clarke, 1996), and ghrelin induced stimulation of growth hormone (GH) release from the anterior pituitary is $[Ca^{2+}]$ dependent (Yamazaki et al., 2004).

The GHSR-1a is expressed in many peripheral tissues and central nuclei, such as the thyroid, myocardium, pancreatic islets, anterior pituitary, adipose tissue, lung, liver, arteries, gonads, hypothalamus, hippocampus, raphe nuclei, and VTA (reviewed in Guan et al., 1991; Ferrini et al., 2009), indicating its diverse range of effects; ghrelin is now known to regulate a wide range of physiological functions including the stimulation of feeding behaviour (Wren et al., 2001; Shintani et al., 2001), reward seeking behaviour (Abizaid et al., 2011; King et al.,

2016; Edwards and Abizaid, 2016; St-Onge, Watts, and Abizaid, 2016; King, Isaacs, O'Farrell, and Abizaid, 2011; Skibicka et al., 2012), stress, anxiety, and depressive behaviours (Patterson et al., 2013; Lutter et al., 2008; Chuang et al., 2011), stimulation of gut motility and gastric acid secretion (Masuda et al., 2000; Asakawa et al., 2001), glucose metabolism regulation (Date et al., 2002; Reed et al., 2008), and the suppression of BAT thermogenesis (Yasuda et al., 2003; Tsubone et al., 2005; Lin et al., 2011). While ghrelin is well known for its ability to act centrally to induce food intake and adiposity, it also has many peripheral actions that contribute to the regulation of energy homeostasis, which are described briefly below. Although the focus here is energy balance, it is important to note that ghrelin also has other peripheral effects including actions on the cardiovascular, immune, reproductive, and bone formation systems that are not described here (for review, see Delporte, 2013).

Peripheral Ghrelin Actions in Energy Balance

Ghrelin plays an important role in glucose homeostasis, and accordingly both ghrelin and the GHSR1a is expressed in pancreatic α -, β -, δ , and ϵ - islet cells (Dezaki et al., 2004; Wierup et al., 2002; DriGruccio et al., 2016). Plasma ghrelin and insulin levels exhibit reciprocal changes (Korbonits et al., 2001; Cummings et al., 2001), which may reflect the ability of ghrelin to inhibit insulin secretion, thereby increasing glucose levels (Broglia et al., 2001; Reimer et al., 2003). In pancreatic β -cells, ghrelin inhibits insulin release through the G_i pathway (Dezaki, Kakei, and Yada, 2007), and infusions of ghrelin also suppress glucose-stimulated insulin release, leading to impaired glucose clearance (Tong et al., 2010). Thus, ghrelin appears to increase glucose by its actions at the level of the pancreas, and is supported by the finding that GOAT inhibition improves glucose clearance by stimulating insulin release (Barnett et al., 2010).

In addition to the pancreas, ghrelin has direct effects on the liver. Ghrelin promotes hepatic gluconeogenesis and also increases triglyceride content, decreasing glucose uptake and fatty acid oxidation in the liver (Rigault et al., 2007; Barazzoni et al., 2004), an effect that occurs via an AMPK mediated pathway (Kola et al., 2005; Barazzoni et al., 2004), but is independent of GH (Sangio-Alvarellos et al., 2009). In GHSR and ghrelin KO animals, hepatic insulin sensitivity is enhanced (Longo et al., 2008; Sun et al., 2006), and ghrelin attenuates the ability of insulin to inhibit hepatic glucose production (Heijboer et al., 2006), suggesting that ghrelin is a positive regulator of liver glucose levels by negatively regulating insulin activity.

Ghrelin induces adiposity via multiple mechanisms; both acyl and desacyl-ghrelin stimulate lipid accumulation in WAT (Rodriguez et al., 2009), in a GH-independent fashion (Sangio-Alvarellos et al., 2009). Ghrelin also promotes the utilization of carbohydrates over the less metabolically efficient use of fat, allowing for more fat deposition (Tschop, Smiley, and Hyman, 2000). In contrast to central ghrelin, in subcutaneous and visceral WAT ghrelin inhibits AMPK activity (Kola et al., 2005), and increases UCP2 mRNA expression (Tsubone et al., 2005). Chronic ghrelin administration increases body fat significantly more in UCP2 KO mice than in WT mice, reflecting a decrease in fat oxidation of UCP2 KO mice (Andrews et al., 2010). This suggests not only that UCP2 plays an important role in metabolism by promoting fat oxidation, but that it also attenuates the ability of ghrelin to induce lipogenesis (Andrews et al., 2010), further reducing fat accumulation. In addition to controlling WAT accumulation, ghrelin modulates BAT activity via the sympathetic nervous system (Yasuda et al., 2003). Central ghrelin administration in rats suppresses sympathetic nerve activity in BAT, reduces energy expenditure and thermogenesis, and attenuates BAT UCP1 and UCP3 mRNA expression (Yasuda et al., 2003; Theander-Carrillo et al., 2006), effects that are independent of ghrelin-induced hyperphagia.

Central Actions of Ghrelin

Central administration of ghrelin potently stimulates food intake, even in satiated animals (Nakazato et al., 2001), although the mechanisms through which ghrelin reaches its receptors in the CNS is an area of ghrelin research that remains highly debated (Edwards and Abizaid, 2017). Most of the research on ghrelin's central effects has focussed on the hypothalamus, however it is apparent that ghrelin also acts in other brain areas to modulate food intake and other behaviours. In particular, the GHSR-1a is expressed within the VTA, primarily on dopamine neurons (Abizaid et al., 2006), and signalling within this pathway is critical for both ghrelin-induced food intake and dopamine release in the nucleus accumbens, which can occur in the absence of a negative energy state. Activation of these neurons by ghrelin leads to an increase in dopamine turnover in the NAc by increasing the ratio of excitatory to inhibitory inputs to these neurons, and leads to an increase in feeding behaviour (Abizaid et al., 2006). Ghrelin activity within this pathway increases reward seeking behaviours, including behaviours associated with obtaining food, sex, and drug rewards (Wise and Volkow, 2005; Abizaid et al., 2006; Zigman et al., 2006; Naleid et al., 2005). Acute or chronic ghrelin administration into the VTA increases the amount of work animals are willing to perform for food in a progressive ratio bar pressing paradigm, as well as the rewarding value of food in a conditioned place preference paradigm (Skibicka et al., 2011; King et al., 2011). King et al., 2011 show that infusions of ghrelin directly into the VTA dose-dependently increases chow intake, and increases bar pressing for chocolate pellets, indicating that ghrelin acts in this area to promote the preference for and motivation to obtain palatable foods.

Ghrelin signaling in the VTA is clearly important for its role in energy balance, however its ability to stimulate non-sated feeding requires activity at other central receptors, since ghrelin

administration to ARC ablated rats fails to induce food intake (Tamura et al., 2002). The ARC is characterized by weak blood-brain barrier formation and is sensitive to the actions of many circulating hormones, and is thus a critical site for their action (Fry and Ferguson, 2010; Tamura et al., 2002). The ARC contains two main neuronal populations controlling energy homeostasis; neuropeptide Y/agouti related peptide/gamma aminobutyric acid (NPY/AgRP/GABA) neurons, which promote food intake, and pro-opiomelanocortin/cocaine and amphetamine-regulated transcript (POMC/CART) neurons, which suppress food intake. The densest expression of central GHSRs are found within the ARC (Zigman et al., 2006), which are primarily localized on NPY/AgRP/GABA neurons (Willesen, Kristensen, and Romer, 1990) and to a lesser extent on POMC/CART neurons (Ferrini et al., 2009). Ghrelin directly regulates NPY/AgRP neurons by increasing their firing rate (Cowley et al., 2003), leading to both increased release of these peptides and increased mRNA expression levels (Kamegai et al., 2001; Wang et al., 2014; Kamegai et al., 2000). NPY induces its orexigenic effects via binding to the G-protein coupled receptors Y1 and Y5 (Marsh, Hollopeter, Kafer, and Palmiter, 1998; Kushi et al., 1998; Nguyen et al., 2012), whereas AgRP is an inverse agonist at the MC3/MC4 receptors (Nijenhuis, Oosterom, Adan, 2001). Genetic deletion of both NPY and AgRP completely blocks ghrelin-induced feeding (Wang et al., 2014), confirming that ghrelin acts via these peptides to induce feeding.

Ghrelin also regulates POMC neurons. POMC is cleaved to produce α -melanocyte stimulating hormone (α -MSH), which has anorectic effects when released from neurons. This melanocortin peptide exerts its effects by binding to central melanocortin receptors 3 and 4 (MC3R and MC4R) within intra- and extra-hypothalamic sites such as the PVN, VMH, DMH, and nucleus of the solitary tract (NTS; Suzuki et al., 2010). Ghrelin negatively regulates the

activity of these POMC/CART neurons, both indirectly by stimulating GABAergic inputs and inhibiting excitatory contacts, and directly by hyperpolarizing them (Cowley et al., 2003; Dietrich and Horvath, 2013), resulting in further promotion of food intake. The effect of ghrelin on NPY/AgRP, but not POMC/CART neurons, is influenced by energy status, as fasting increases the number of ghrelin-labelled NPY/AgRP neurons, but has no effect on ghrelin-labelled POMC/CART (Schaeffer et al., 2013).

In addition to acting directly in the ARC to affect NPY/AgRP and POMC/CART signaling, the ability of ghrelin to regulate these peptides arises, at least in part, from the VMH (Lopez et al., 2008). Inhibition of AMPK locally in the VMH blocks not only ghrelin-induced feeding, but also ghrelin-induced increases in ARC NPY/AgRP (Lopez et al., 2008). This suggests that ghrelin-sensitive VMH AMPK neurons are able to modulate NPY/AgRP neurons in the ARC via presynaptic connections (Yang et al., 2011; Lopez et al., 2008). It has been suggested that the hypothalamic AMPK pathway may also be important for the ability of ghrelin to act centrally to modulate other metabolic actions, such as glucose homeostasis (Li et al., 2012). In contrast to its inhibitory actions on AMPK in WAT, ghrelin acts in the brain to activate the AMPK pathway. Both ghrelin and AMPK are upregulated during severe caloric restriction (Lopez et al., 2016), and both VMH AMPK and ghrelin are important in maintaining hypoglycemia during negative energy balance states (McCrimmon et al., 2004). In addition, GHSR NPY/AgRP neurons of the ARC project to the paraventricular nucleus of the hypothalamus (PVN), and innervation by GHSR positive terminals has also been detected in the dorsomedial, lateral, and paraventricular nuclei of the hypothalamus (Cowley et al., 2003), suggesting that ghrelin may act in other hypothalamic structures to regulate metabolic functions.

In the PVN, ghrelin is thought to act on GHSRs to promote carbohydrate intake and decrease fat intake, as pharmacological blockade of ghrelin receptors locally in the PVN leads to an increase in fat over carbohydrate intake (Patterson et al., 2013). In *ad libitum* animals, viral knockdown of the GHSR in the VMH increases food intake and body weight (Merkenstein et al., 2014). In animals on a restricted feeding paradigm, VMH GHSR knockdown attenuates running wheel activity but does not affect food intake, leading to a mitigation of body weight loss (Merkenstein et al., 2014). Ghrelin is stimulatory in VMH neurons (Yanagida et al., 2008), and stimulation of VMH neurons reduces food intake (Stenger, Fournier, and Bielajew, 1991), thus knockdown of VMH GHSR may lead to an overall inhibition of the VMH, promoting food intake. Overall, it is clear that ghrelin has distinct metabolic actions in the hypothalamus that vary as a function of location and energy status.

The DMH

In addition to the other hypothalamic nuclei discussed in previous sections, in mice there is also moderate expression of the GHSR1a in the DMH, and low expression in the DMH of rats (Zigman et al., 2006), but the phenotype of these DMH GHSR positive neurons remains unknown. The DMH is located in the mediobasal hypothalamus immediately dorsal to the VMH, and has long been associated with energy homeostasis. Early experiments in sheep suggested that the DMH is an orexigenic nucleus, as stimulation of the nucleus led to hyperphagia (Larsson, 1954). This was later confirmed by the finding that lesions of the DMH causes not only reduced meal size, leading to hypophagia, but also hypodipsia and reduced linear growth (Bernardis, 1970; Bellinger et al., 1986). It is now known that in addition to the modulation of ingestive behaviour and linear growth, the DMH plays a critical role in other physiological functions,

including the control of body temperature and thermogenesis (DiMicco and Zaretsky, 2006; Cao and Morrison, 2006; Jeong et al., 2015), circadian rhythms (Chou et al., 2003; Mieda et al., 2006), endocrine and behavioural responses to stress (DiMicco et al., 2001), sympathetic activity (Horiuchi, McDowall, and Dampney, 2006), and anxiety (Shekhar, 1993). More recent research has focused on understanding the mechanisms by which the DMH modulates these functions, such as the chemical phenotype of DMH neurons, afferent and efferent projections, and receptor expression within this area, which are depicted in figure A and discussed in more detail below.

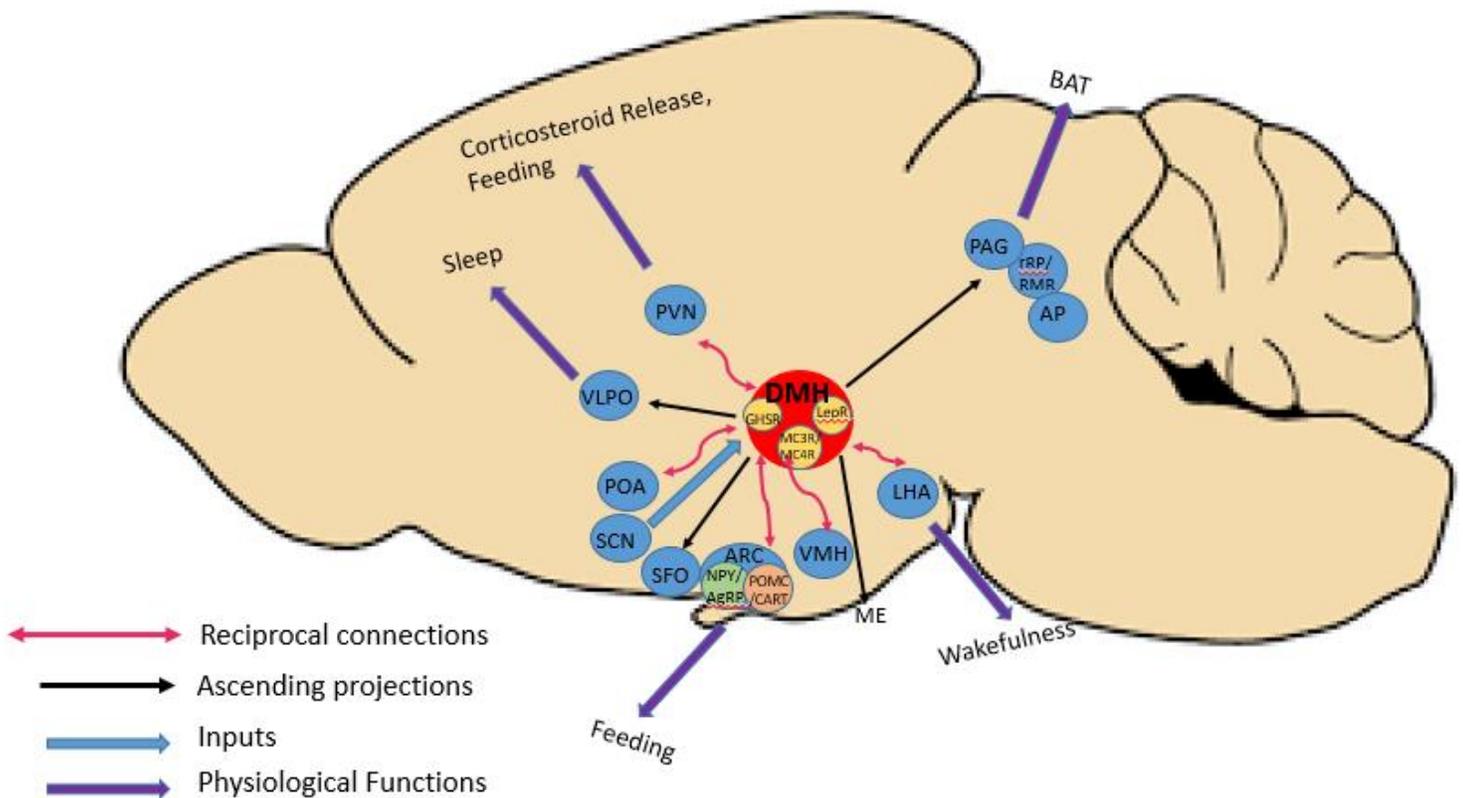


Figure A. Simplified depiction of the projections to and from the DMH. Note that many connections are omitted for simplicity.

GHSR; growth hormone secretagogue receptor, **LepR**; leptin receptor, **MC3R/MC4R**; melanocortin 3 and melanocortin 4 receptors, **BAT**; brown adipose tissue, **NPY/AgRP**; neuropeptide Y/agouti-related peptide, **POMC/CART**; pro-opiomelanocortin/cocaine and amphetamine regulated transcript,

ARC; arcuate nucleus, **AP**; area postrema, **DMH**; dorsomedial hypothalamus, **LHA**; lateral hypothalamic area, **ME**; median eminence, **PAG**; periaqueductal gray, **POA**; preoptic area, **PVN**; paraventricular nucleus, **rRP/RMR**; rostral raphe pallidus/rostral medullary raphe, **SCN**; suprachiasmatic nucleus, **SFO**; subfornical organ, **VMH**; ventromedial hypothalamus

DMH Control of Food Intake

The DMH expresses many feeding related peptides, including neuropeptide Y (NPY), cholecystokinin (CCK), corticotrophin-releasing factor (CRF), galanin (GAL), and glucagon-like peptide 2 (GLP-2), and various peptide receptors, including CCK1Rs, melanocortin receptor 3 and 4 (MC3R/MC4R), leptin receptors (LepR), and the ghrelin receptor, making this area well equipped to mediate energy balance (Bi, 2007). DMH neurons express high levels of GABAergic markers (Draper et al., 2010; Tappaz, Brownstein, and Kopin, 1977), as well as glutamatergic markers (Ziegler, Cullinan, and Herman, 2002), however there are also populations of tyrosine hydroxylase positive cells (a marker for dopamine and norepinephrine synthesis) (van den Pol, Herbst, and Powell, 1984), choline acetyl transferase positive cells (a marker for acetylcholine) (Rao et al., 1987), and serotonin neurons (Savedra et al., 1974). DMH GABAergic neurons, which are hyperpolarized by leptin and depolarized by glucose (Otgon-Uul et al., 2016), promote food intake by inhibiting the PVN, a site of anorexigenic signals (Otgon-Uul et al., 2016). In addition, the DMH also projects to the nucleus of the solitary tract (NTS), and the vagus dorsal motor nucleus (DMV) (Moran and Kinzig, 2004), areas known to integrate information from the gastrointestinal system to control satiation, thus providing other possible pathways by which the DMH controls ingestive behaviour.

Evidence suggests that NPY signaling in the DMH may be the central mediator of its effects on energy balance control. In contrast to the ARC, where *Npy* expression levels are leptin-dependent and are increased following an acute fast, *Npy* expression in the DMH only increases following chronic food restriction (Bi et al., 2003; Draper et al., 2010; Bi, Kim, and Zheng, 2012). Nevertheless, studies show that this system is important for the regulation of energy homeostasis, as adeno-associated virus (AAV)-mediated overexpression of NPY in the

DMH causes increased food intake and body weight in both chow and high-fat diet fed animals (Yang et al., 2009), while viral mediated knockdown ameliorates the hyperphagia and elevated body weight observed in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Yang et al., 2009). One important regulator of NPY in the DMH is CCK1 (Bi et al., 2001). Indeed, CCK1R knockouts show increased DMH *Npy* expression, which is not co-localized with CCK1R expression (Bi et al, 2001; Bi et al., 2004), while in chow fed, non-obese animals, NPY neurons co-express CCK1R (Bi et al., 2004). This suggests that CCK acts on NPY within the DMH to regulate its expression, and that the lack of CCK1R and the subsequent elevation in *Npy* expression contributes to the hyperphagia and obesity observed in this animal model. This is supported by the finding that intra-DMH CCK downregulates DMH *Npy* expression and decreases food intake over a 22 hour period (Chen et al., 2008; Blevins, Stanley, and Reidelberger, 2000). Thus, the DMH is critical for the control of food intake, and relies on NPY containing cells, a feature shared with ghrelin-induced food intake.

DMH Control of Thermoregulation

Body weight is maintained by a balance between energy intake and energy expenditure and there is evidence that the DMH may contribute to both sides of this equation because in addition to modulating food intake, it is also a site of control of thermogenesis. Brown adipose tissue (BAT) is a mitochondria-rich adipocyte store that possesses thermogenic properties, and is a key regulator of body temperature and energy expenditure in small animals (Cannon and Nedergaard, 2004). In recent years, it has gained considerable attention as a potential target for the treatment of obesity because of its detection in adult humans (Cypess et al., 2009; Virtanen et al., 2009; van Marken Lichtenbelt et al., 2009; Ouellet et al., 2012). Animal studies have revealed

that the central control of BAT thermogenesis occurs primarily at the level of the hypothalamus and brainstem (Richard, 2015). BAT dissipates energy in the form of heat via uncoupling protein 1 (UCP1)-mediated non-shivering thermogenesis (NST), and through this process can burn up to half of ingested triglycerides and 75% of ingested glucose (Nedergaard et al., 2011). The thermogenic capacity of BAT is highly dependent on activity of the sympathetic nervous system (SNS; Bartness et al., 2010; Bartness and Ryu, 2015), the release of noradrenaline (NA), and binding of NA to β 3-adrenergic receptors within BAT (Bachman et al., 2002). The use of the pseudorabies virus for retrograde transneuronal tracing has allowed the identification of the central regions targeting BAT; these include brain areas involved in the regulation of body temperature (lateral parabrachial nucleus; NTS (Morrison et al., 2014; Nakamura and Morrison, 2008), as well as those involved in the regulation of energy balance, including the ARC (Haynes et al., 1999), preoptic area (POA) (Yoshida et al., 2009; Nakamura, 2011), ventromedial hypothalamus (VMH; Musatov et al., 2007; Lopez et al., 2010), lateral hypothalamus (LH; Berthoud et al., 2005; Tupone et al., 2011), PVN (Madden and Morrison, 2009), and DMH (Ushikubi et al., 1998; Scammell et al., 1996; Lazarus et al., 2007).

The DMH appears to be a positive regulator of BAT activity, as stimulation or disinhibition of DMH neurons increases sympathetic nerve activity to BAT, and elevates local BAT and core body temperature (DiMicco and Zaretsky, 2007; Morrison and Nakamura, 2011), while inhibition of DMH neurons prevents BAT thermogenesis (Morrison et al., 2008). Anatomically, the DMH is a relay centre between the POA, an integrator of central and peripheral thermal signals, and neurons in the rostral medullary raphe (RMR; Yoshia et al., 2009), which affect BAT thermogenesis via glutamatergic inputs to sympathetic premotor neurons (SPNs; Nakamura et al., 2004). POA warm sensitive GABAergic neurons are inhibited

during cold exposure, which then allow DMH thermogenic neurons to stimulate sympathetic nerve activity to BAT via the RMR (Yoshida et al., 2009).

While the neurochemicals involved in DMH mediated control of BAT are unknown, a few likely candidates are being investigated, including leptin and NPY. Leptin, which acts on central LepRs to decrease food intake and increase energy expenditure, has gained considerable attention when considering the mechanisms of DMH mediated control of BAT. Administration of leptin increases UCP1 expression in BAT (Scarpace and Matheny, 1998), an effect that is dependent on the MC4R pathway and an intact SNS, as α -MSH antagonists or surgical denervation of the SNS blocks leptin-induced expression of UCP1 (Satoh et al., 1998; Williams et al., 2003). The thermogenic actions of leptin are dependent on the DMH, as blocking LepRs in the DMH blocks the effects of leptin on iBAT temperature (Enriori et al., 2011). While leptin is a positive regulator of BAT thermogenesis, NPY appears to be a negative regulator of BAT activity, as central administration suppresses sympathetic activity in iBAT (Egawa et al., 1991). The central actions of NPY on iBAT activity may also occur at the level of the DMH, as DMH NPY knockdown increases iBAT UCP1 and stimulates white into brown adipocyte transformation of inguinal fat (Chao et al., 2011). The up or downstream pathways mediating the actions of DMH NPY activity on brown fat are not well understood, however DMH NPY neurons do not contain LepRs (Bi et al., 2003). Together these data suggest that DMH-mediated increases in BAT activity is influenced both by central peptides such as NPY, and peripheral signals of energy balance like leptin, however whether ghrelin signalling within this area can also modulate BAT thermogenesis is not yet known.

DMH Control of Circadian Rhythms

Body temperature and food intake show clear circadian rhythms and the DMH is also an important mediator of circadian outputs (Mieda et al., 2006). It has been suggested that the DMH integrates circadian information from both direct and indirect inputs from the suprachiasmatic nucleus (SCN; Thompson and Swanson, 1998; Chou et al., 2003), with information about energy status from the periphery to modulate a wide range of functions (Saper et al., 2005). The SCN, the master clock that synchronizes circadian rhythms (Moore and Eichler, 1972), sends efferent projections to relatively few forebrain nuclei, but its densest projections terminate in the subparaventricular zone and the DMH (Watts and Swanson, 1987; Stephan, Berkley, and Moss, 1981). In contrast, the DMH has widespread efferent projections, suggesting that it is an important output centre for the circadian system (Chou et al., 2003). The densest DMH outputs are to regions within the hypothalamus, such as the PVN (Elmquist et al., 1998), but it also projects to regions that control sleep and wakefulness (Chou et al., 2003). The DMH sends GABAergic projections to the ventrolateral preoptic nucleus (sleep-promoting area), and a glutamatergic thyrotropin-releasing hormone (TRH) projection to the lateral hypothalamus (LHA; wake-promoting area; Chou et al., 2003). This suggests that the DMH controls circadian rhythms of arousal by promoting wakefulness. This is supported by evidence showing that excitotoxic lesions of the DMH affects a range of circadian rhythms, including feeding and locomotor activity, wakefulness, and corticosteroid levels (Chou et al., 2003). The DMH is densely innervated by GABAergic projections from the SCN, and local administration of the GABA agonist muscimol into the DMH blocks the normal rise in plasma melatonin during the dark phase via reduced sympathetic outflow to the pineal gland (Kalsbeek et al., 1996), suggesting that GABAergic projections from the SCN regulate sympathetic activity via the

DMH. Thus, the DMH is an important area for regulating a wide-range of circadian rhythms entrainable by light.

In addition to light entrainable rhythms, the DMH is also critical for food entrainable rhythms (Mieda et al., 2006). It is known that the timing of food availability affects behavioural and physiological rhythms, and that animals on a restricted feeding schedule will show anticipatory activity prior to the presentation of their scheduled meal. Further, when the pattern of this scheduled meal exposure and the light cycle are in conflict, the rhythm will reflect that of the scheduled meal, suggesting that food availability patterns override light-dark patterns. However, the site of this food-entrainable oscillator (FEO) remains a debate. The DMH is a likely candidate in this regard, due to its ability to receive and send information about both circadian rhythms and energy status. Animals under RFS show increases in DMH mPer1 and mPer2 oscillation amplitudes (Mieda et al., 2006). When this RFS is placed in the middle of the day, the highest expression of DMH Fos shifts to occur in the light phase, compared to *ad libitum* animals who show the greatest expression of DMH Fos in the middle of the dark phase (Angeles-Castellanos et al., 2004; Saper et al., 2005). This suggests that the timing of the outputs from the DMH is modulated by food availability, supporting the hypothesis that the DMH is an important integrator of both circadian and feeding information. Indeed, cell-specific lesions of the DMH blocks the expression of food entrainable circadian rhythms, including wakefulness, locomotor activity, and body temperature (Gooley et al., 2006). However, these paradigms involve food restricting the animals and presenting their only access to food in a limited time window. Whether the DMH is important for anticipation to a scheduled meal or treat when animals remain on a background of *ad libitum* food availability is not known. Further, little is known about ghrelin's functions in the DMH.

Ghrelin in the DMH

Chronic food restriction or intraperitoneal injections of ghrelin produces a marked increase in FOS immunoreactivity in the DMH (Blum et al., 2009; Kobelt et al., 2008), suggesting that peripheral ghrelin may act in the DMH. To date, there is only one published experiment investigating the role of GHSR signaling in the DMH on any physiological functions. Merkenstein et al., 2014 employed an AAV method in order to selectively knockdown the GHSR in the DMH in both *ad libitum* and restricted feeding schedule (RFS) rats. The authors indicate that while knockdown of the GHSR in the DMH does not affect food intake or body weight in *ad libitum* animals, it does attenuate running wheel activity in both the light and dark phase (Merkenstein et al., 2014). In RFS rats, DMH GHSR knockdown attenuates weight loss, likely by reducing running wheel activity primarily in the light phase, without affecting food intake or meal patterns (Merkenstein et al., 2014). In addition, the authors found that onset and amplitude of food anticipatory activity was reduced following GHSR knockdown (Merkenstein et al., 2014).

The Ventral Premammillary Nucleus

The ventral premammillary nucleus has a higher density of ghrelin receptors than the DMH, lies just posterior to the DMH, and has gained considerable attention for its role in integrating metabolic cues for in the regulation of reproduction (Leshan and Pfaff, 2014). The neurochemistry and neuroanatomical connectivity of the PMV indicates the importance of this region in integrating metabolic and reproductive cues to alter physiology (figure B). The PMV has reciprocal projections with sexually dimorphic brain nuclei, such as the POA, medial amygdala (MeA), and lateral septal nucleus (LS; Nakano et al., 1997; Spratt and Herbison, 2002; Cabalcante et al., 2006; Canteras, Simerly, and Swanson, 1992). It also receives and sends signals to many feeding centres, including the arcuate nucleus of the hypothalamus (ARC), VMH, PVN, and lateral hypothalamic area (LHA), among others (Leshan and Pfaff, 2014; Canteras et al., 1992; Csaki et al., 2000; Hahn and Coen, 2006). PMV neurons express the satiety neuropeptide CART, and receptors for cannabinoids (CB1R), leptin, insulin, and ghrelin (Guan et al., 1997; Gustafson et al., 1997; Kishi et al., 2007; Marcus et al., 2001; Perello et al., 2012; Zigman et al., 2006). Notably, approximately half of PMV neurons express the leptin receptor and are activated by leptin administration (Elmqvist et al., 1998; Caron et al., 2010), and its role in regulating reproduction within this region is well defined (Leshan and Pfaff, 2014). Aside from leptin, which is known for its role in coordinating reproduction and energy status (Ahima et al., 1996), the PMV is responsive to other factors involved in the control of reproduction, such as sex hormones (Simerly et al., 1990), and substance P (Akesson, 1993).

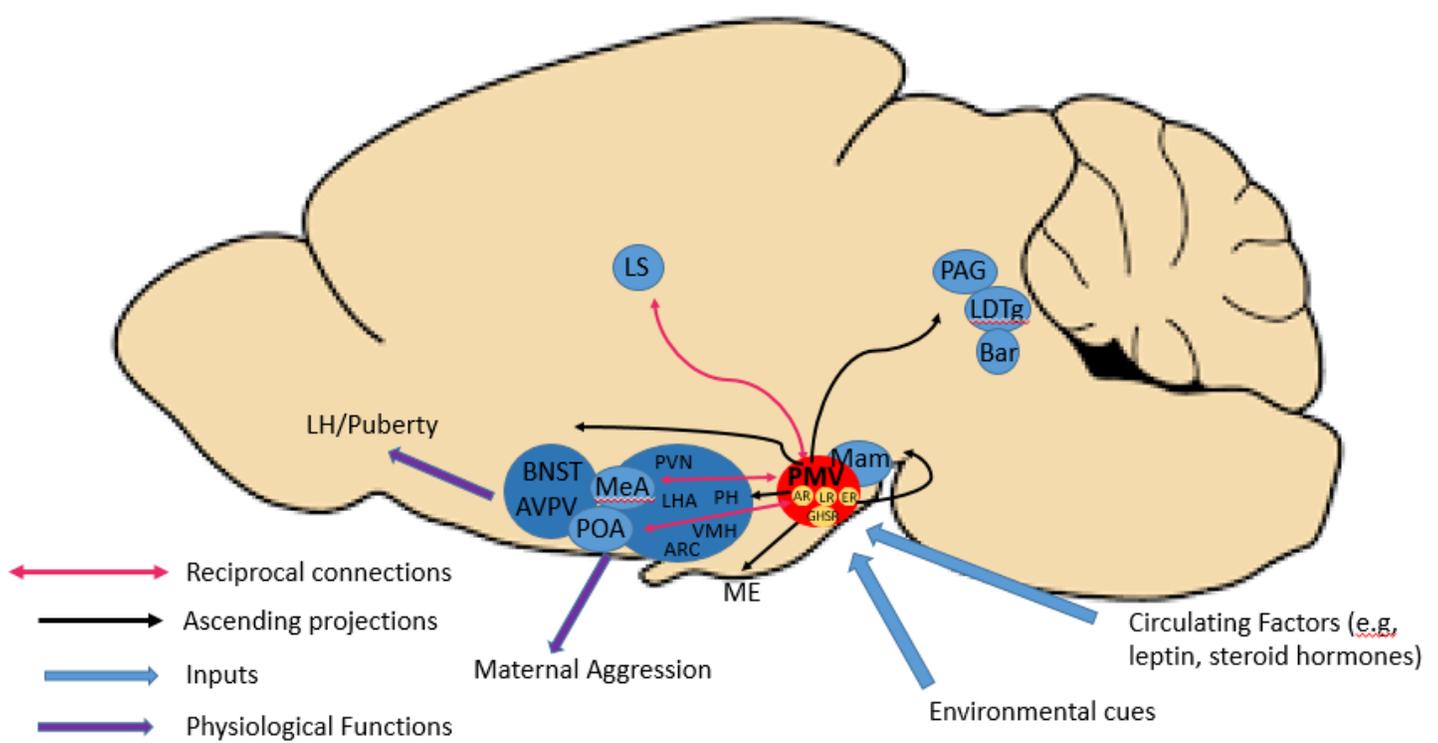


Figure B. Simplified depiction of the projections to and from the PMV. Note that many connections are omitted for simplicity.

AR; androgen receptor, **ER;** estrogen receptor, **GHSR;** growth hormone secretagogue receptor, **LR;** leptin receptor, **LH;** luteinizing hormone,

ARC; arcuate nucleus, **AVPV;** anteroventral periventricular nucleus, **Bar;** Barrington's nucleus, **BNST;** bed nucleus of the stria terminalis, **LDTg;** laterodorsal tegmental area, **LHA;** lateral hypothalamic area, **LS;** lateral septal nucleus, **Mam;** mammillary bodies, **ME;** median eminence, **PAG;** periaqueductal gray, **PH;** posterior hypothalamus, **POA;** preoptic area, **PMV;** ventral premammillary nucleus, **PVN;** paraventricular nucleus, **VMH;** ventromedial hypothalamus

While the PMV expresses numerous receptors related to energy balance and projects to many feeding-related nuclei, lesion studies have ruled out the PMV as a key site for the regulation of energy balance (Donato et al., 2009; Shiriashi and Mager, 1980a,b). 2-deoxyglucose lesions of the PMV induce hypoglycemia and hypothermia, and it has been suggested to be an area important for thermoregulation (Shiriashi and Mager, 1980). Almost 30 years later, Donato and colleagues (2009) found that bilateral excitotoxic lesions of the PMV did not affect body weight or food intake, but did dampen both a leptin-induced and preovulatory luteinizing hormone (LH) surges. This suggests that the PMV is necessary for coordinating the phasic neuroendocrine changes characteristic of a cycling female, with metabolic information, to alter the normal

neuroendocrine events in response to changes in energy balance, but not for energy balance itself. However, whether or not the PMV becomes important following endogenous changes in metabolic factors such as those that occur from fasting or diet-induced obesity has not been investigated. Denroche and colleagues (2016), used a transgenic line of leptin receptor knockout mice, where LepR is selectively inactivated only in the LHA and PMV ($LepR^{flox/flox}Syn-Cre$ mice), and showed that animals without leptin signaling in these two nuclei are resistant to diet-induced obesity when placed on a HFD (Denroche et al., 2016). They also noted that these animals have elevated leptin, insulin, and glucagon secretion, but did not differ in body weight, food intake, or energy expenditure (Denroche et al., 2016). While this suggests that leptin signaling in the LHA/PMV may be important for the regulation of insulin and glucose, it is unclear whether these effects are driven primarily by the LHA, PMV, or a combination of both regions (Denroche et al., 2016). It is also unclear whether these effects are a result of altered neuronal programming during development, since leptin signaling in the hypothalamus is important for normal development of the hypothalamic feeding circuits (Bouret et al., 2012). In addition to leptin, the PMV is responsive to many other energy balance signals, including CART, insulin, and ghrelin, but their relevance in modulating reproduction, energy homeostasis, or any other physiological functions is not yet known. Indeed, CART neurons are stimulated in the PMV of male rats following exposure to female odours (Cavalcante, Bittencourt, and Elias, 2006), and these neurons express the leptin receptor, therefore it is possible that all nutritional signals of the PMV are important only for the control of reproduction rather than energy balance itself.

Aims and Hypotheses

The overall aim of this thesis is to investigate how exogenously stimulating the GHSR1a, or blocking endogenous binding of ghrelin, locally within the DMH or PMV alters energy balance in male mice. These areas were chosen primarily because they both express GHSR1a. The DMH has been implicated in a number of functions in which ghrelin has also been shown to play a role, including food intake and energy expenditure, as well as food anticipatory activity. Although data available, thus far, suggests that the PMV does not play a major role in energy balance *per se*, it is clearly a target for peripheral metabolic signals. Targeting this nucleus with the same regimen of agonists and antagonists used in the DMH allowed us to evaluate the site specificity of any effects observed in the DMH. In addition, the wide range of metabolic measures used in these studies allowed for a more nuanced assessment of metabolic effects of ghrelin signalling in the PMV.

A secondary aim of these studies was to evaluate the effects of manipulating GHSR1a signalling in the DMH to anticipatory activity to a scheduled treat. Previous experiments have shown that intra-DMH GHSR1a knockdown reduces onset and amplitude of anticipatory activity in restricted fed animals without affecting general rhythmicity (Merkenstein et al., 2014). There is also ample evidence that free feeding rodents show anticipatory activity to scheduled presentation of a highly palatable treat (Mistlberger and Rusak, 1987; Gallardo et al., 2012) and as noted above, ghrelin increases intake of palatable foods in sated animals. However, whether ghrelin acts in the DMH to modulate anticipatory behaviour to such treats has not yet been investigated and this issue was addressed in the current studies by providing daily scheduled 4h presentation of a high fat diet.

Since ghrelin is an orexigenic peptide that can act centrally to induce food intake and increase body weight, and that the DMH is an orexigenic nucleus, we hypothesize that intra-DMH ghrelin would produce increases in food intake and body weight, whereas infusions of a GHSR antagonist would attenuate food intake and body weight. Since central ghrelin administration also slows peripheral glucose clearance and reduces energy expenditure, and the DMH is an important mediator for sympathetic nerve activity, we hypothesize that intra-DMH ghrelin will similarly slow glucose clearance and attenuate energy expenditure, whereas infusions of a GHSR antagonist would improve glucose clearance and elevate energy expenditure. We also hypothesize that since central ghrelin promotes the preference for a carbohydrate diet over a high fat diet in previously exposed animals, that intra-DMH ghrelin would similarly lead to an increase in chow intake in animals habituated to a 4h access to a high fat diet, while a GHSR antagonist would attenuate the preference for chow. Further, we hypothesize that even in animals given *ad libitum* access to standard laboratory chow, presentation of scheduled high fat diet exposure will induce anticipatory activity in all animals, and that this effect will be attenuated by intra-DMH administration of a GHSR antagonist.

Since the PMV is not thought to be an important regulator of energy balance or circadian rhythms, and that PMV lesioned animals do not display alterations in food intake or body weight (Donato et al., 2009), we hypothesize that intra-PMV ghrelin will not affect food intake, body weight, diet preference, energy expenditure, or anticipatory activity. However, because previous lesion experiments have demonstrated that the PMV is an important glucoregulatory area, we expect that chronic infusions of ghrelin will limit glucose clearance, whereas infusions of a GHSR antagonist will promote its clearance.

General Methods

Subjects

Adult male mice (C57BL/J6, 20-25 grams) were obtained from Charles Rivers farms (St. Constant, Quebec). All mice were individually housed in clear Plexiglas 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 *ad 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 am and 12:30 pm each day. 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. All procedures were approved by Carleton University Animal Care Committee according to the guidelines of the Canadian Council of Animal Care (CCAC).

Timeline

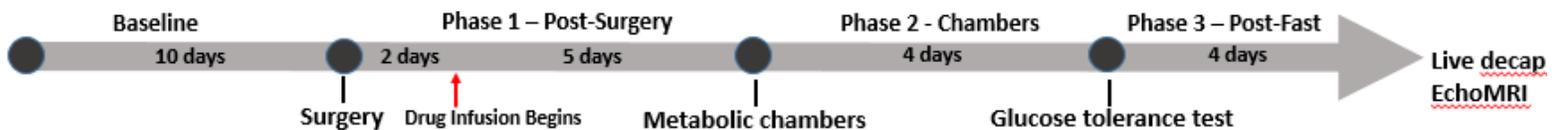


Figure C. Depiction of experimental timeline and experimental days of each phase.

Surgical procedure

Following the baseline period, 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*, (2013). 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 Prividine 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 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 an osmotic minipump of assigned treatment (Alzet Mini-Osmotic Pump – Model 1004; flow rate 0.11 μ L/h for 28 days) was implanted into the dorsomedial hypothalamus or ventral premammillary nucleus. Stereotactic coordinates relative to bregma were DMH: AP 1.7mm, ML 0.4mm, and DV 5.25 mm, PMV: AP 2.6mm, ML 0.3mm, and DV 5.4mm. 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 Medicam (1 mg/kg) was injected 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 hours after surgery.

Metabolic Chambers

Procedures used were similar to those described in Patterson *et al.*, (2012). 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 hours 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 minutes for 48 hours. 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: $\frac{CV(of\ RER)*VO_2(\frac{ml}{h})}{body\ weight\ (kg)}$ and is also calculated by the software. Only the last 24 hours of this period were used for analyses, as the first 24 hours are typically used to allow acclimation. Upon the completion of this test, mice were returned to their home cages for another period of acclimation.

Glucose Tolerance Test

At least 4 days after returning to their home cages, mice underwent a glucose tolerance test which provides a measure of rate of glucose clearance from the 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 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 20% glucose (2g of glucose per kg of body mass). Blood was taken at 15, 30, 60, and 120 minutes after injection and 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 carcasses were analyzed for fat content using the EchoMRI Body Composition Analyzer EF-020 (Houston, Texas, www.echomri.com).

Cannula placements

Brains were extracted and stored in 4% PFA in PB for at least 72 hours 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 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.

Statistical Analysis

Food intake and body weight across the entire period of drug administration were expressed as change from baseline and the effect of drug condition was investigated using a 2-way ANOVA with drug treatment as the independent variable and days as the within group variable. Further, inspection of this data revealed clear effects of the manipulations of house in the metabolic chambers as well as the fast imposed on the animals as part of the GTT paradigm, thus we separated the experimental period into 3 distinct phases (see figure C). Phase 1 (post-surgery) is defined as the first 5 days following the beginning of drug infusion, which starts 48 hours post-surgery. Phase 2 (chambers) is defined as the 2 days where animals were housed in

the metabolic chambers following the 7 day post-surgery phase, as well as the 5 recovery days following removal from the chambers. Phase 3 (post-fast) is defined as the day following the overnight fast (for glucose tolerance test), and the 3 recovery days following the fast prior to decapitation; data from the fast day was excluded from the analysis for phase 3. Change across each phase is measured as a function of change from the prior. Where appropriate, data was analyzed within each phase. Furthermore, in order to account for changes in total caloric intake or body weight with the experimental manipulations on RER or energy expenditure, where appropriate we used ANCOVAs with lean mass, total caloric intake, or chow intake as a covariate.

Caloric intake, body weight, metabolic chamber measures (RER, locomotor activity, VCO₂, VO₂, energy expenditure), and 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, Fisher's LSD 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 Fisher's LSD post-hoc analyses were performed where appropriate.

Experiment 1 (Pilot study): Dose-Response Curve of JMV2959 for the DMH and PMV

Groups and Drugs

Following the baseline period, mice were assigned to one of six experimental drug treatment groups: PMV-vehicle (isotonic saline, n=7), PMV-low dose antagonist (JMV2959 2.0 µg/day, n=7), PMV-high dose antagonist (JMV2959 20 µg/day, n=7), DMH-vehicle (isotonic saline, n=7), DMH-low dose antagonist (JMV2959 2.0 µg/day, n=7), DMH-high dose antagonist (JMV2959 20 µg/day, n=7). JMV2959 was purchased from EMD Millipore, Calbiochem[®], and was dissolved in isotonic saline in the doses described.

Experiment 2: Ghrelin, JMV2959, and Saline in the DMH

Groups and Drugs

Following the baseline period, mice were assigned to one of three experimental drug treatment groups: DMH-vehicle (n=12), DMH-ghrelin (n=12), DMH-JMV2959 (n=12). JMV2959 was obtained from EMD Millipore, Calbiochem[®], and was dissolved in isotonic saline (2µg/day). Ghrelin was obtained from Peptides International, and was also dissolved in isotonic saline (10µg/day). The remainder of the methods were identical to that described above with one exception, as a result of animal care policy changes, blood was obtained from a tail nick, rather than a tail snip during the glucose tolerance test.

Experiment 3: Ghrelin, DLYS-GHRP6, and Saline in the PMV

Subjects

Following the baseline period, mice were assigned to one of three experimental drug treatment groups: PMV-vehicle (n=10), PMV-ghrelin (n=10), PMV- DLYS-GHRP-6 (n=10). DLYS-GHRP-6 and ghrelin were both obtained from Peptides International and dissolved in isotonic saline (18.6 µg/day and 10µg/day, respectively).

Results

Experiment 1: Pilot Study

Determination of JMV2959 Dose

In order to determine an appropriate dose of JMV2959 for infusion within the DMH and PMV in mice, we conducted a pilot study using 2 doses of JMV2959 aimed at either the DMH or PMV. 4 out of the 42 mice used did not recover from surgery and were thus sacrificed and removed from all analyses. A further 9 animals were removed because of incorrect cannula placement. Results are summarized below in table 1. Total caloric intake and body weight are represented as a change from baseline period. Our final group numbers were DMH-vehicle (n=4), DMH-low dose antagonist (n=3), DMH-high dose antagonist (n=5), PMV-vehicle (n=5), PMV-low dose antagonist (n=5), PMV-high dose antagonist (n=3).

Table 1

Total caloric intake (TCI; calories), body weight (g), RER, VO2 (ml/h), locomotor activity (beam breaks), glucose clearance (area under the curve; AUC) of animals treated with ghrelin, saline, or JMV2959 2µg/day or JMV2959 20µg/day administered into either the DMH or PMV (mean +/- SEM).

Measure	Treatment					
	Saline	DMH		Saline	PMV	
		JMV 2µg	JMV20µg		JMV 2µg	JMV 20µg
TCI	1.94 +/- 1.32	1.70 +/- 1.69	1.69 +/- 1.47	1.34 +/- 0.66	3.33 +/- 0.59*	4.83 +/- 0.76**
Body weight	0.37 +/- 0.26	-1.01 +/- 0.56*	-0.36 +/- 0.41**	0.51 +/- 1.14	0.02 +/- 0.69	-0.06 +/- 0.30
RER	0.78 +/- 0.03	0.84 +/- 0.03	0.82 +/- 0.03	0.84 +/- 0.03	0.83 +/- 0.02	0.81 +/- 0.02
VO2	113.63 +/- 4.20	116.82 +/- 4.85	117.88 +/- 4.20	108.65 +/- 2.59	112.33 +/- 2.32	115.99 +/- 2.99
Activity	795.71 +/- 156.31	990.62 +/- 180.49	951.48 +/- 156.31	590.79 +/- 65.61	523.99 +/- 73.24*	963.68 +/- 84.57**
AUC (GTT)	52.89 +/- 2.23	46.78 +/- 4.88*	46.88 +/- 2.16*	45.79 +/- 2.11	43.22 +/- 1.88	43.57 +/- 2.43

*p<0.05 (from saline) **p<0.05 from JMV 2µg

Because both doses of JMV2959 within the DMH produced significant effects on body weight, and 2µg produced a greater effect than 20µg, we chose the lower 2µg dose for our second experiment.

Experiment 2: Effects of manipulating ghrelin receptor signaling within the DMH

To determine whether GHSR signaling in the DMH is important for energy balance or contributes to anticipation of a scheduled treat, saline, ghrelin, or JMV2959 were infused into the DMH for 18 days. 4 animals did not recover from surgery or were sacrificed and were thus removed from all analyses. Further, 5 animals were removed due to incorrect cannula placements, and 1 outlier was removed from all analyses. Thus, 25 animals were included in the final analysis, with the following group numbers: saline (n=9), ghrelin (n=10), JMV2959 (n=6).

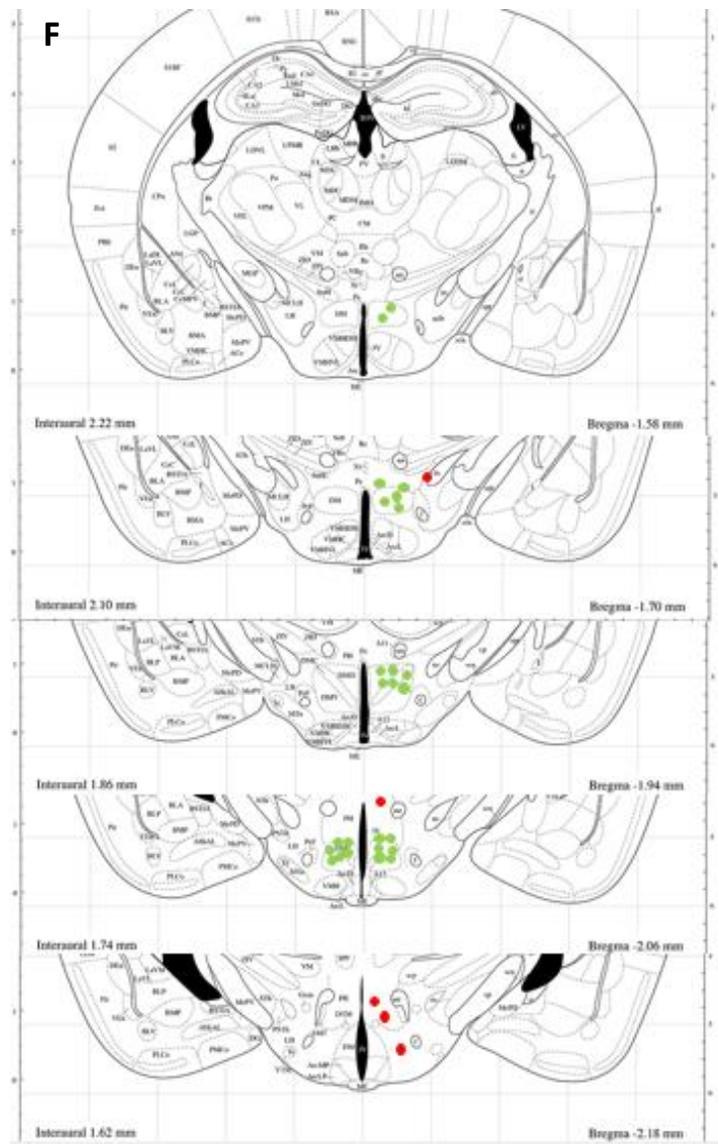


Figure D, E, and F. Figures D and E: Images taken of representative cannula placements for location within the dorsomedial hypothalamus. **Figure F.** Summary of cannula placements for experiment 2. The red dots represent placements falling outside the DMH and were therefore excluded from all analyses. The green dots represent those that fall within or just above the DMH and were therefore included in the analysis.

Exogenous stimulation of the GHSR in the DMH increases body weight

Body Weight Change

Animals receiving ghrelin treatment gained significantly more weight than saline treated animals across the 16 days of the experiment (figure 1A), resulting in a significant main effect of group ($F(2,22)=3.952$, $p<0.05$), partial $\eta^2=0.264$, post-hoc comparison saline vs ghrelin ($p<0.05$). There was no significant difference between JMV2959 treated and both ghrelin treated and saline treated animals. All mice gained weight across time (figure 1B), (significant main effect of time ($F(6,439,141.665)=100.1$, $p<0.0001$, partial $\eta^2=0.820$), but as expected exposure to the metabolic chambers and overnight fasting suppressed body weight increases. A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

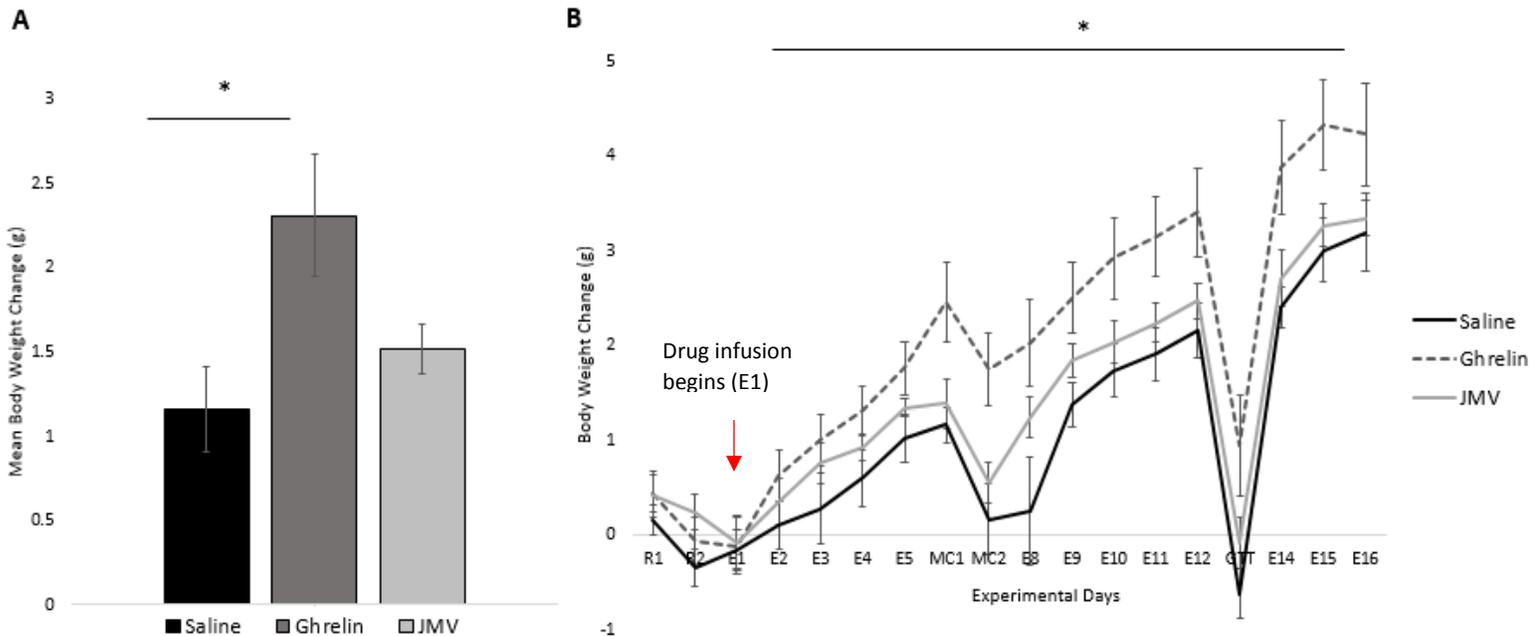


Figure 1A. Mean daily body weight change (g) (+/- SEM). Figure 1B. Mean daily body weight change (g) from day 1 to day 16 of the experimental period.

Inhibition of endogenous ghrelin binding to the GHSR in the DMH reduces total caloric intake through a reduction in chow but not high fat diet intake

Chow Intake Change

Animals receiving JMV2959 ate significantly less chow than both saline and ghrelin treated animals (figure 2A), resulting in a significant main effect of group ($F(2,22)=4.254$, $p<0.05$), partial $\eta^2=0.279$, post-hoc comparisons JMV2959 vs ghrelin ($p<0.05$), JMV2959 vs saline ($p<0.05$). There was no significant difference between ghrelin treated animals and saline treated animals. Additionally, chow intake differed across time (figure 2B), resulting in a significant main effect of time ($F(7.041,154.911)=28.434$, $p<0.0001$, partial $\eta^2=0.564$, but this effect was driven primarily by the absence of food intake during the GTT fast and the post-fast increase in food intake. A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

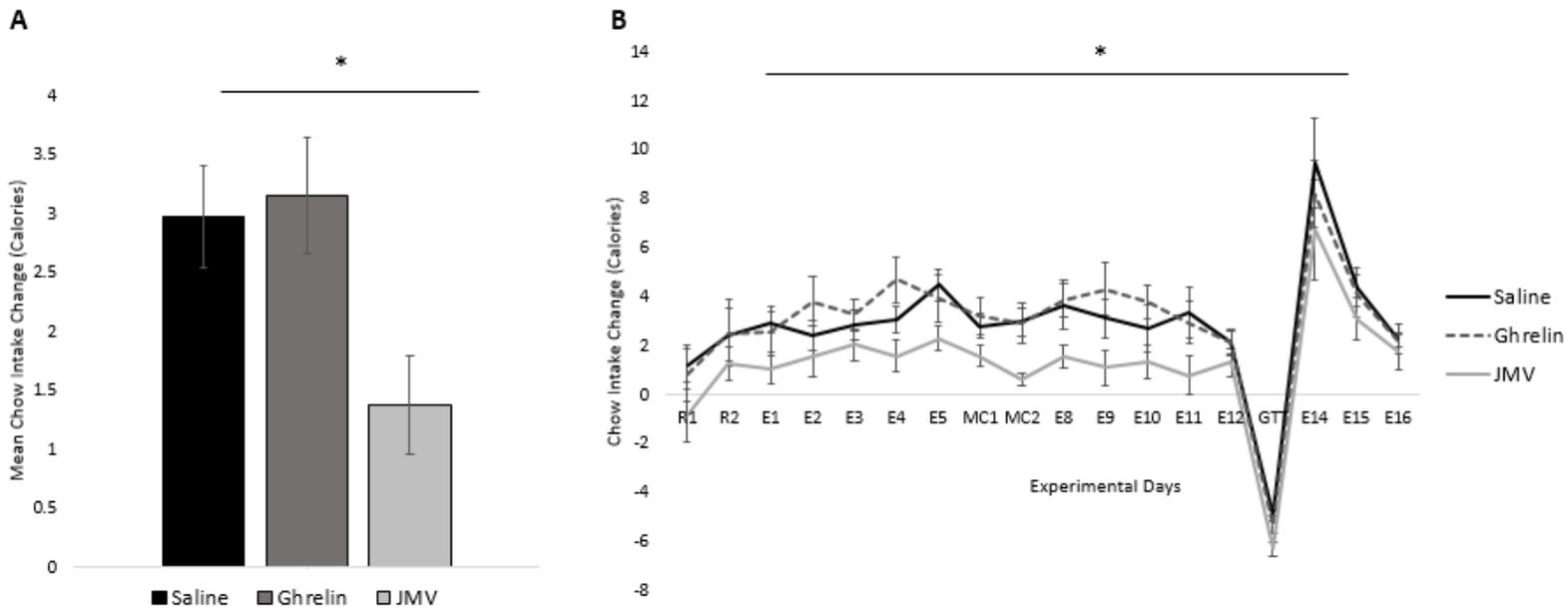


Figure 2A. Mean daily chow intake change (calories) (+/- SEM). Figure 2B. Mean daily chow intake change (calories) from day 1 to day 16 of the experimental period.

High Fat Diet Intake Change

High fat diet intake increased across time (figure 3), resulting in a significant main effect of time ($F(11.505,253.112)=65.28$, $p<0.0001$, partial $\eta^2=0.748$, however a similar pattern was seen in all groups, and neither the main effect for group nor the group X time interaction effect was significant.

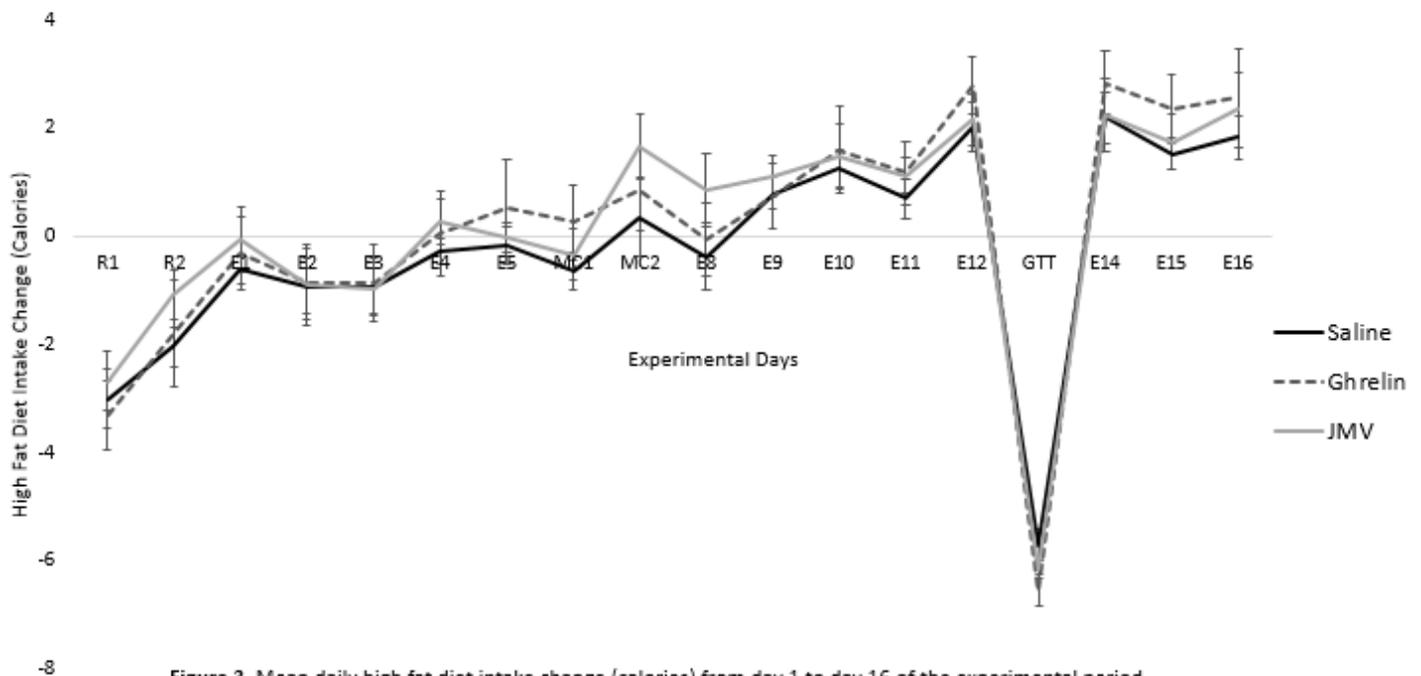


Figure 3. Mean daily high fat diet intake change (calories) from day 1 to day 16 of the experimental period.

Total Caloric Intake Change

Animals receiving JMV2959 ate significantly fewer calories than ghrelin treated animals (figure 4A), resulting in a significant main effect of group ($F(2,22)=5.057$, $p<0.05$), partial $\eta^2=0.315$, post-hoc comparisons JMV2959 vs ghrelin ($p<0.05$), JMV2959 vs saline ($p<0.05$). There was no significant difference between ghrelin treated animals and saline treated animals. Additionally, total caloric intake differed across time (figure 4B), significant main effect of time ($F(6.979,153.538)=76.99$, $p<0.0001$, partial $\eta^2=0.778$, which seemed to reflect primarily the

overnight fast imposed on the mice prior to the GTT and the post-fast rise in food intake. A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

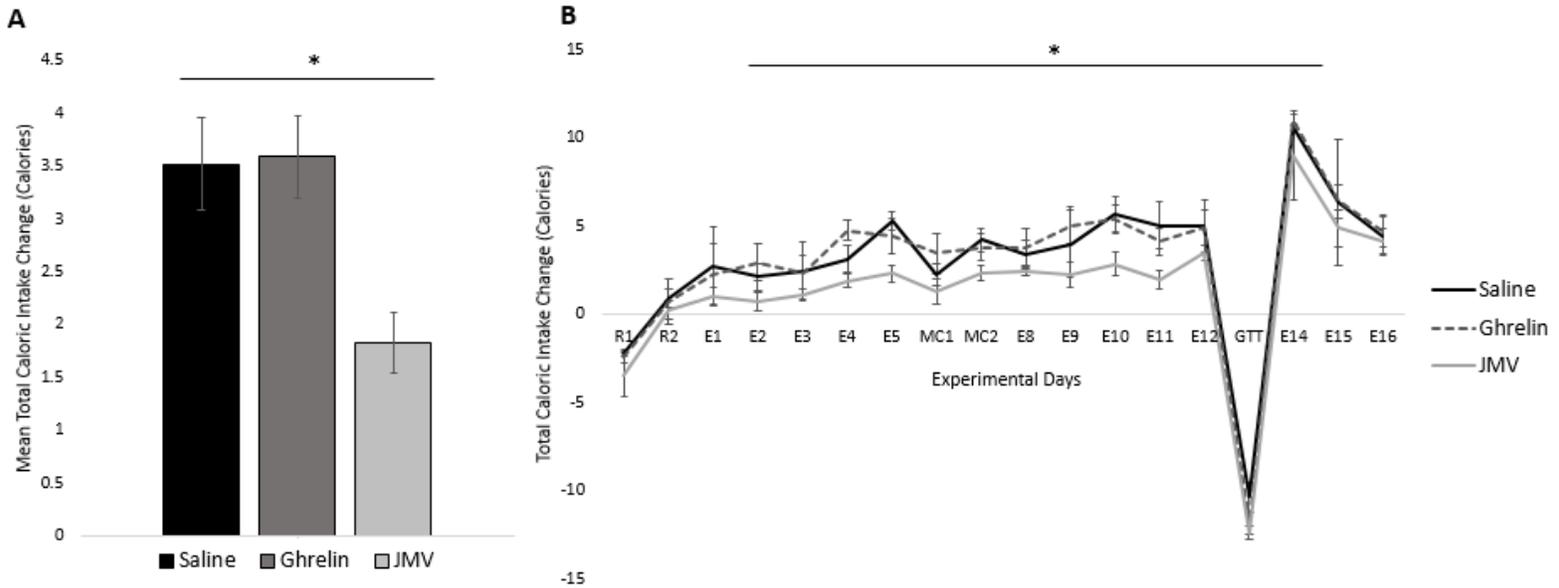


Figure 4A. Mean daily total caloric intake change (calories) (+/- SEM). Figure 4B. Mean daily total caloric intake change (calories) from day 1 to day 16 of the experimental period.

Exogenous stimulation of the GHSR in the DMH increases body weight during phase 1

Phase 1 Body Weight Change

Daily and overall change in body weight for phase 1 is shown in figures 5A and 5B, respectively. Analysis of these data revealed no significant main effect of drug ($F(2,22)=2.05$, $p>0.05$), partial $\eta^2=0.157$. However, as expected, animals receiving ghrelin treatment gained significantly more weight than saline treated animals and this was supported by a significant planned comparison between saline and ghrelin groups ($p<0.05$). Additionally, body weight increased across time (figure 5B), resulting in a significant main effect of time ($F(4,88)=58.931$, $p<0.0001$, partial $\eta^2=0.728$). A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

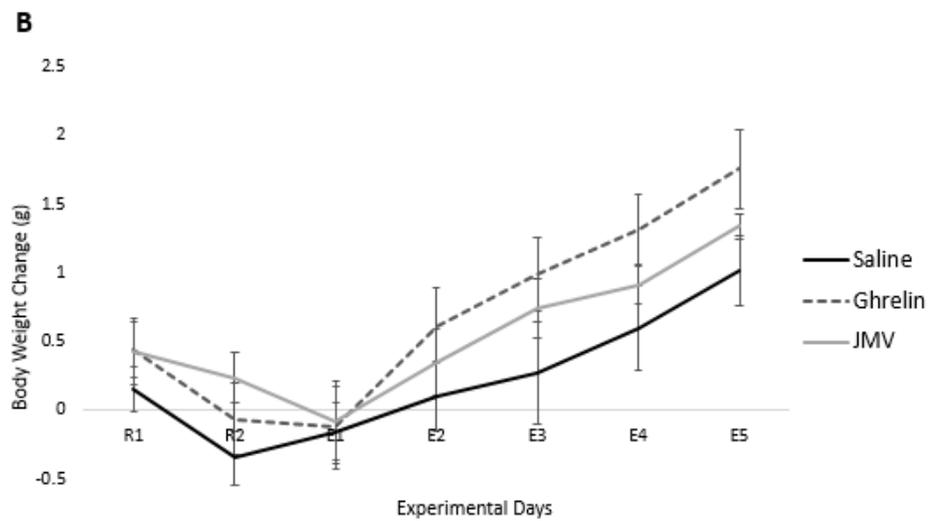
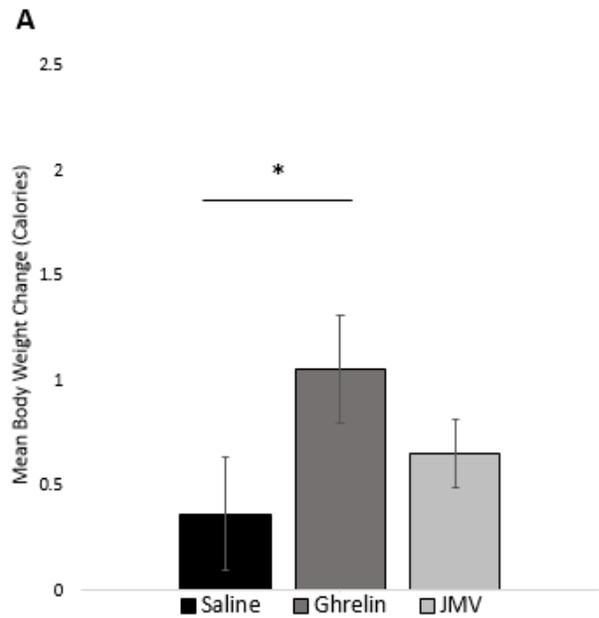


Figure 5A. Mean daily body weight change (g) (+/- SEM). Figure 5B. Mean daily body weight change (g) from day 1 to day 5 of the experimental period.

Phase 1 Chow Intake Change

Daily change in chow intake for phase 1 is shown in figure 6. Analysis of these data revealed no significant main effect of drug ($F(2,22)=2.588, p>0.05$), partial $\eta^2=0.190$, or time ($F(4,88)=2.11, p>0.05$, partial $\eta^2=0.088$).

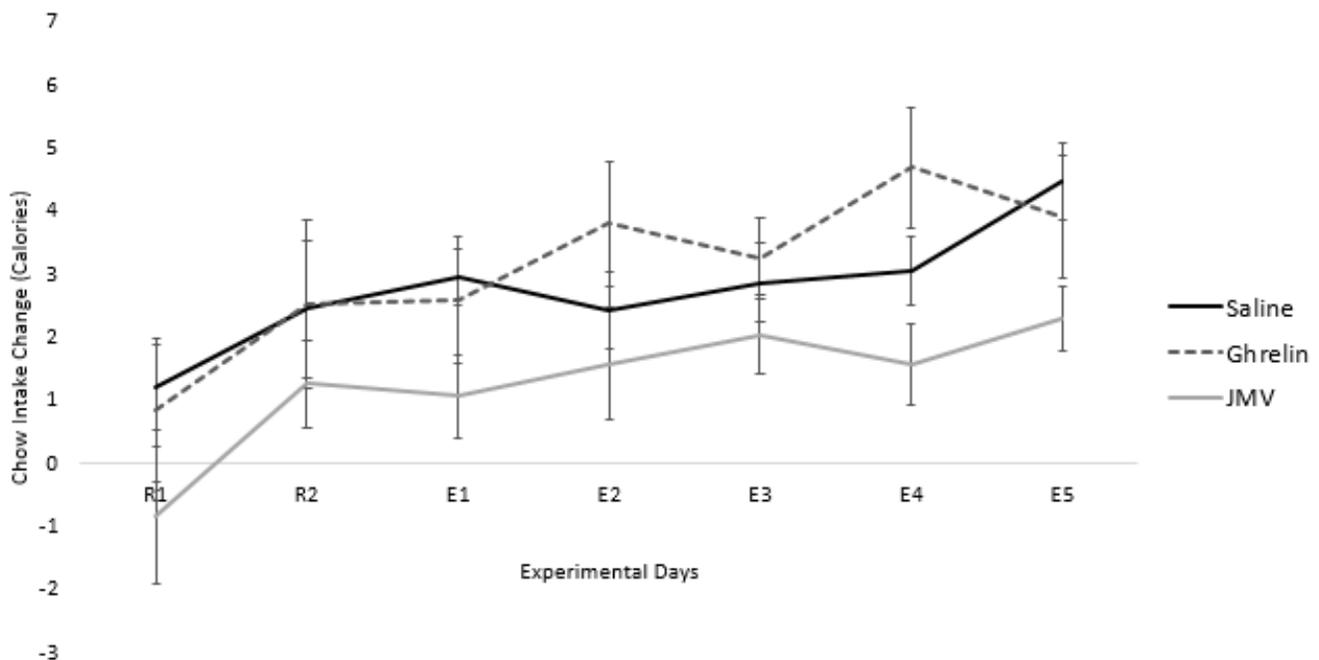


Figure 6. Mean daily chow intake change (g) from day 1 to day 5 of the experimental period.

Phase 1 Total Caloric Intake Change

Daily change in total caloric intake for phase 1 is shown in figure 7. While the main effect of drug did not attain significance ($F(2,22)=3.080$, $p=0.06$), partial $\eta^2=0.219$. As a result of our hypothesis and the large body of literature supporting the orexigenic effects of ghrelin, we did a planned comparison between ghrelin vs JMV2959 ($p<0.05$), indicating that as expected, JMV2959 treated animals ate fewer total calories than ghrelin treated animals. This effect is primarily driven by a decrease in chow intake, coupled with a slight, non-significant decrease in high-fat diet intake (data not shown; see appendix for stats). Additionally, total caloric intake change differed across time, resulting in a significant main effect of time ($F(4,88)=4.384$, $p<0.005$, partial $\eta^2=0.165$). A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

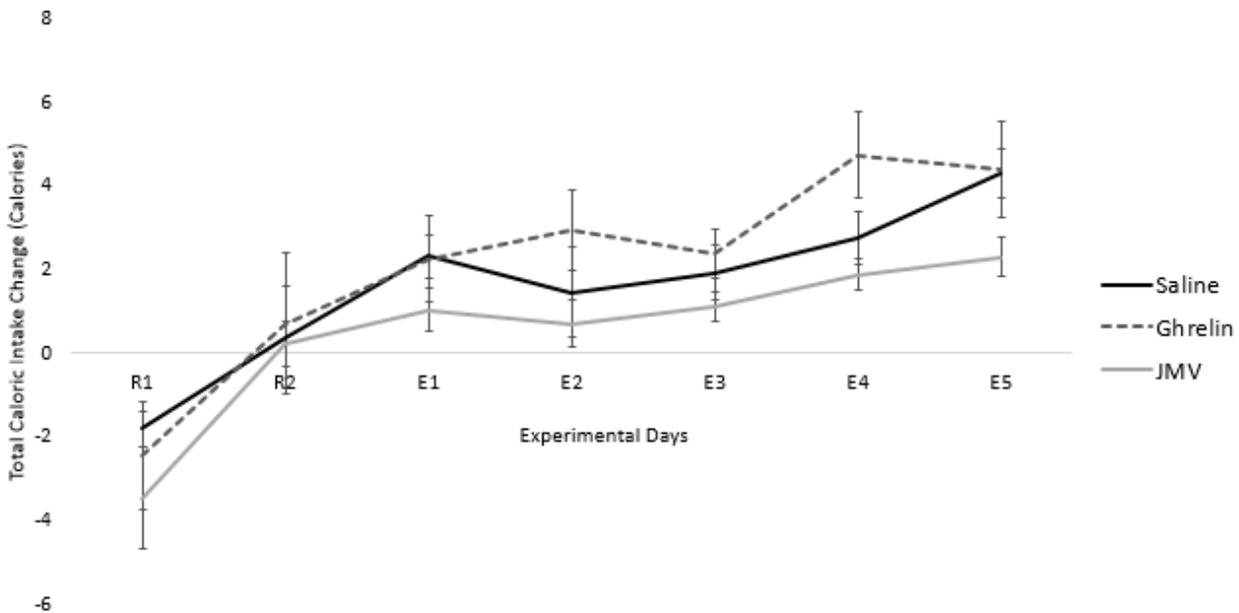


Figure 7. Mean daily total caloric intake change (g) from day 1 to day 5 of the experimental period.

Exogenous stimulation of the GHSR in the DMH increases body weight during phase 2

Phase 2 Body Weight Change

Animals receiving ghrelin treatment gained significantly more weight during phase 2 than saline treated animals (figure 8A), resulting in a significant main effect of group ($F(2,22)=3.483$, $p<0.05$), partial $\eta^2=0.240$, post-hoc comparisons saline vs ghrelin ($p<0.05$).

There was no significant difference between JMV2959 treated and either ghrelin treated or saline treated animals. Additionally, body weight changed across time (figure 8B), resulting in a significant main effect of time ($F(2.309,50.808)=34.473$, $p<0.0001$, partial $\eta^2=0.610$, and as expected exposure to the metabolic chambers suppressed body weight increases. A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

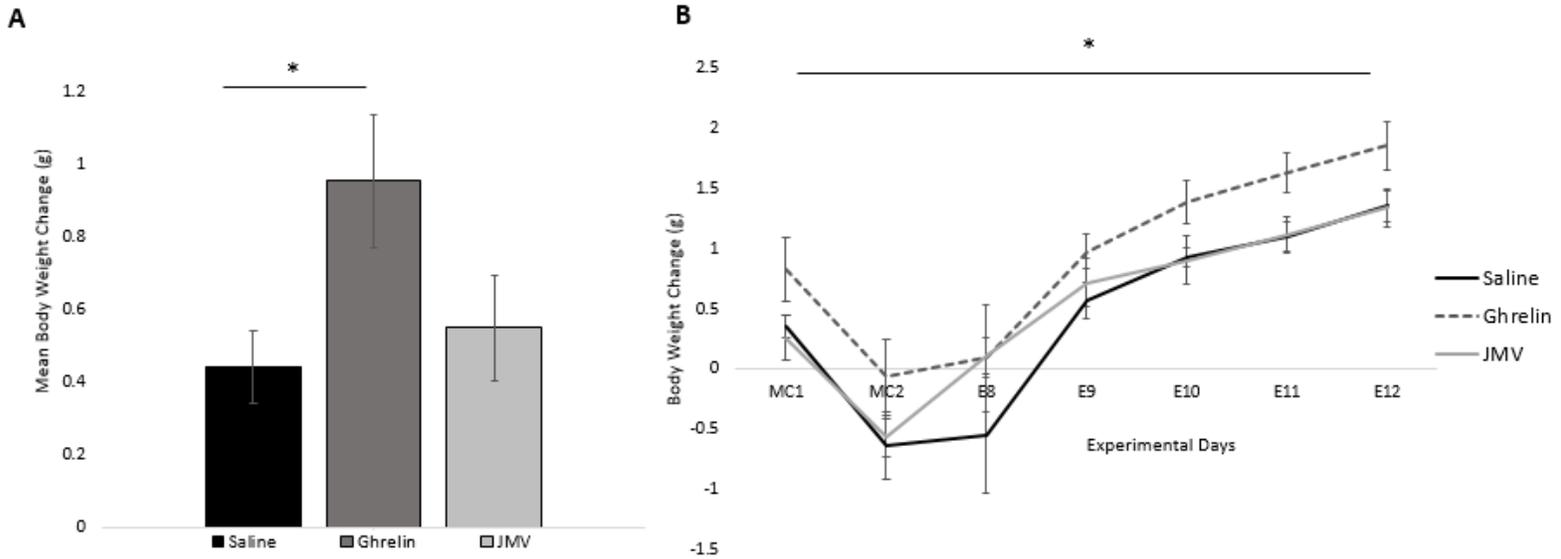


Figure 8A. Mean daily body weight change (g) (+/- SEM). Figure 8B. Mean daily body weight change (g) from day 6(MC1) to day 12 of the experimental period.

Phase 2 Chow Intake Change

Daily change in chow intake for phase 2 is shown in figure 9. The overall repeated measures ANOVA revealed no significant main effect of drug ($F(2,22)=0.043$, $p>0.05$), $\eta^2=0.053$, or time ($F(5.364,118.007)=1.223$, $p>0.05$, partial $\eta^2=0.053$).

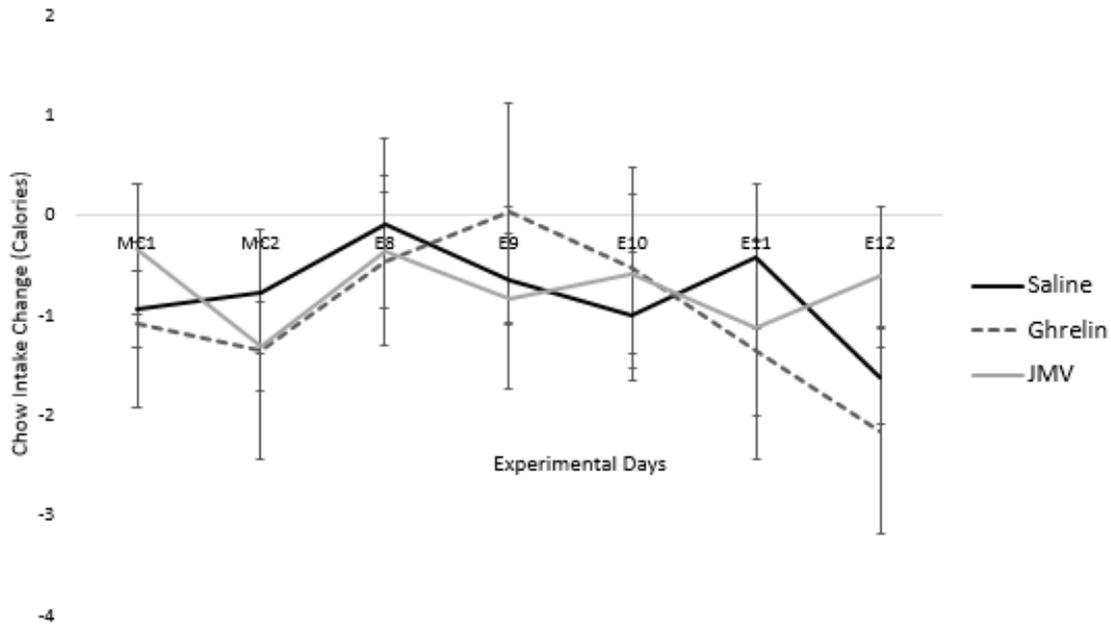


Figure 9. Mean daily chow intake change (calories) from day 8(MC1) to day 12 of the experimental period.

Phase 2 Total Caloric Intake Change

Daily change in total caloric intake for phase 2 is shown in figure 10. Analysis of these data revealed no significant main effect of drug ($F(2,22)=0.139$, $p>0.05$), partial $\eta^2=0.012$, or time ($F(5.437,119.614)=2.065$, $p>0.05$, partial $\eta^2=0.086$).

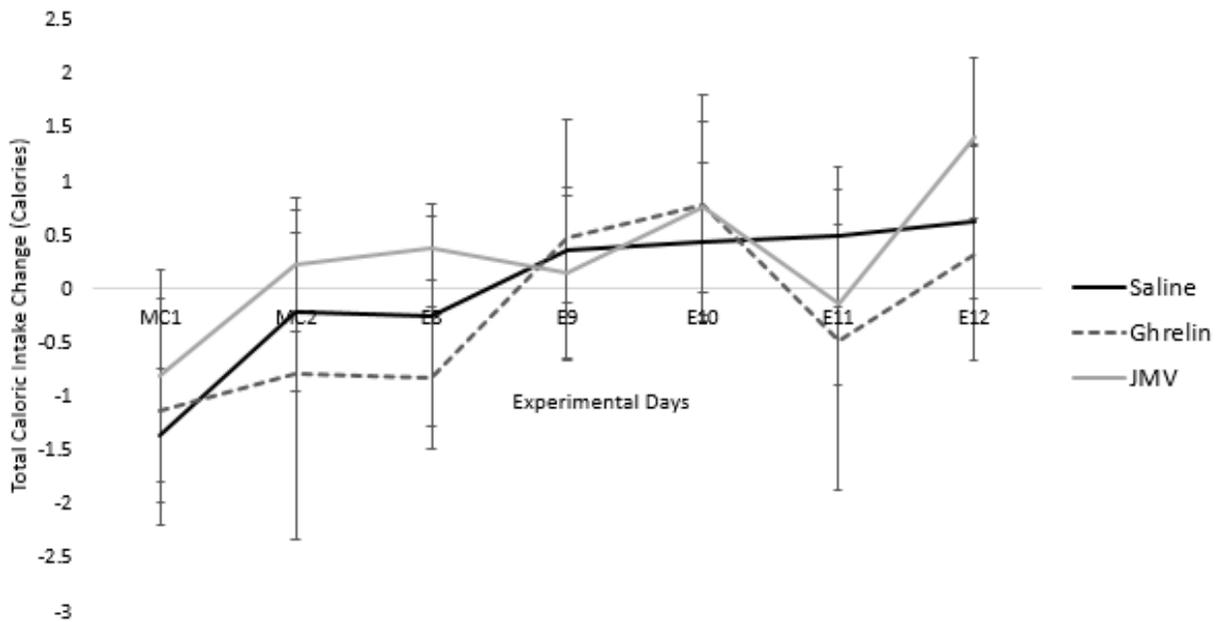


Figure 10. Mean daily total caloric intake change (calories) from day 6(MC1) to day 12 of the experimental period.

Manipulating ghrelin receptor signaling in the DMH does not affect body weight or caloric intake post-fast

Phase 3 Body Weight Change

Daily change in body weight for phase 3 is shown in figure 11. Analysis of these data revealed no significant main effect of drug ($F(2,22)=0.63$, $p>0.05$), partial $\eta^2=0.054$.

Additionally, body weight increased across time ($F(1.37,30.13)=14.976$, $p<0.001$, partial $\eta^2=0.405$, however a similar pattern was seen in all groups, and the group X time interaction effect was not significant.

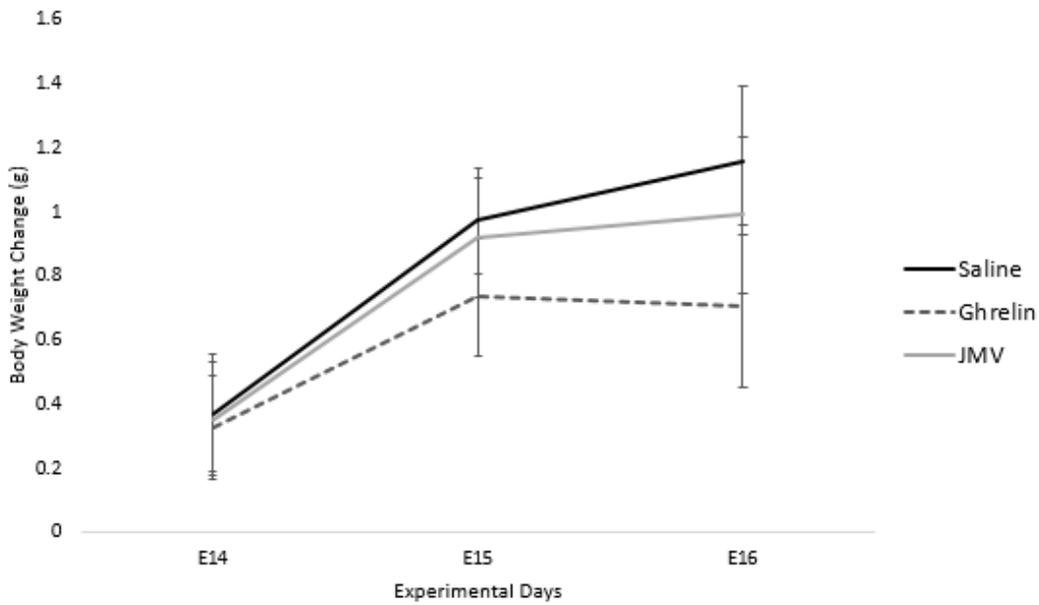


Figure 11. Mean daily body weight change (g) from experimental day E14(post-fast) to day 16

Phase 3 Total Caloric Intake Change

Daily change in body weight for phase 3 is shown in figure 12. Analysis of these data revealed no significant main effect of drug ($F(2,22)=0.049$, $p>0.05$), partial $\eta^2=0.004$.

Additionally, total caloric intake changed across time ($F(1.134,29.45)=24.379$, $p<0.0001$, partial $\eta^2=0.526$, which seemed to reflect primarily the post-fast rise in food intake. A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

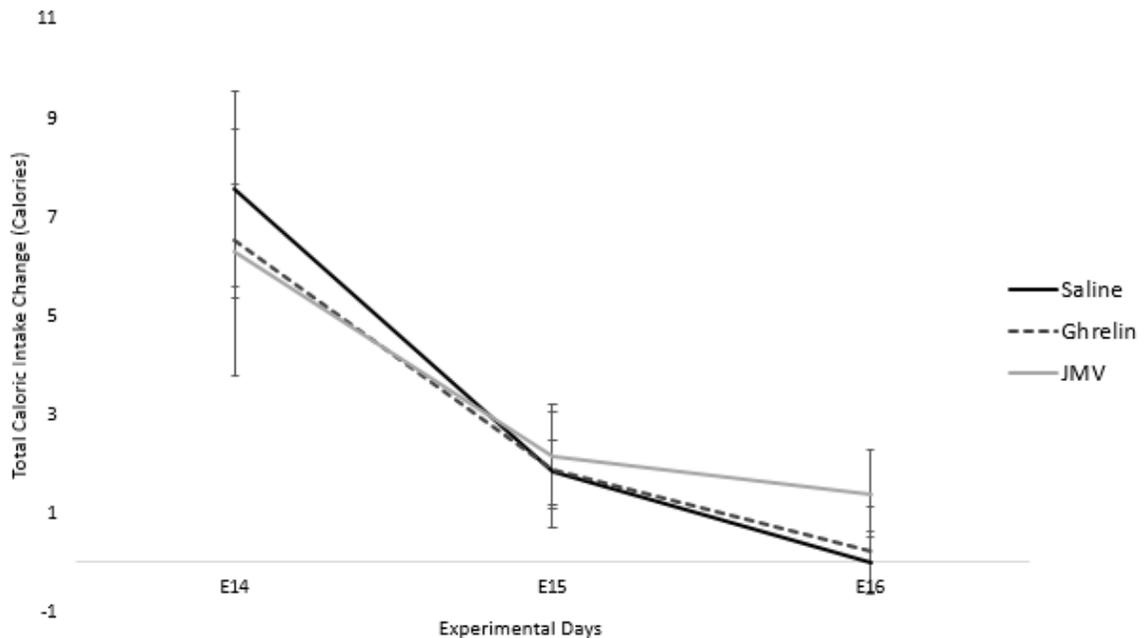


Figure 12. Mean daily total caloric intake change (calories) from experimental day 15(GTT) to day 18

Inhibition of endogenous ghrelin binding in the DMH promotes carbohydrate metabolism during the dark phase of the light-dark cycle

Respiratory exchange ratio

Individual 30 minute RER values across a 24 hour period is shown in figure 13.

Analysis of these data revealed a significant main effect of drug during the light phase (figure 14A) ($F(2,22)=4.638$, $p<0.05$), partial $\eta^2=0.297$, post-hoc JMV2959 vs saline ($p<0.05$), JMV2959 vs ghrelin ($p<0.05$), but not the dark phase (figure 14B) ($F(2,22)=1.183$, $p>0.05$, partial $\eta^2=0.097$). Additionally, RER changed across time during both the light and dark phase, resulting in a significant main effect of time (light phase; ($F(14.153, 311.356)=19.779$, $p<0.0001$, partial $\eta^2=0.473$, dark phase; ($F(10.383, 228.426)=5.52$, $p<0.001$, partial $\eta^2=0.201$ but this is primarily due to the expected circadian pattern of fuel utilization. A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

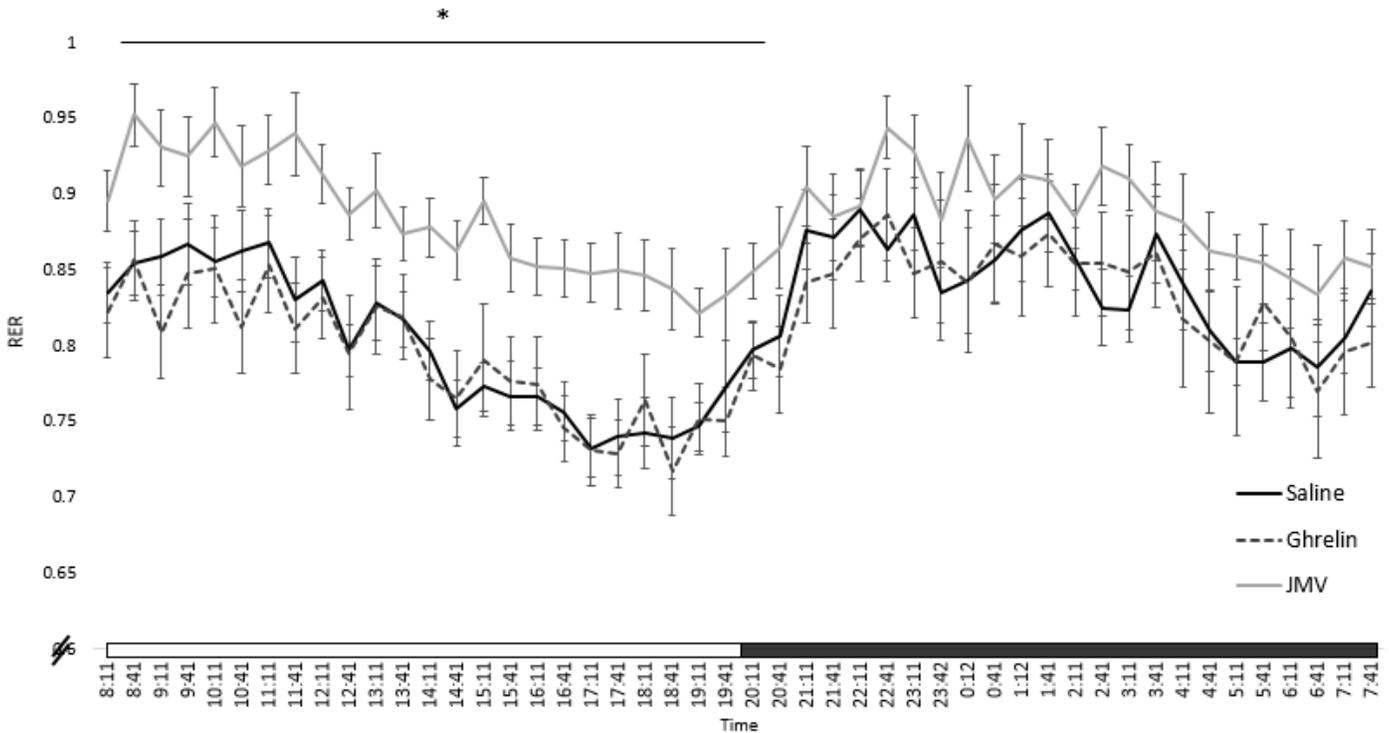


Figure 13. Respiratory exchange ratio at 30 minute intervals across a 24 hour period. White bar indicates the light phase, black bar indicates dark phase.

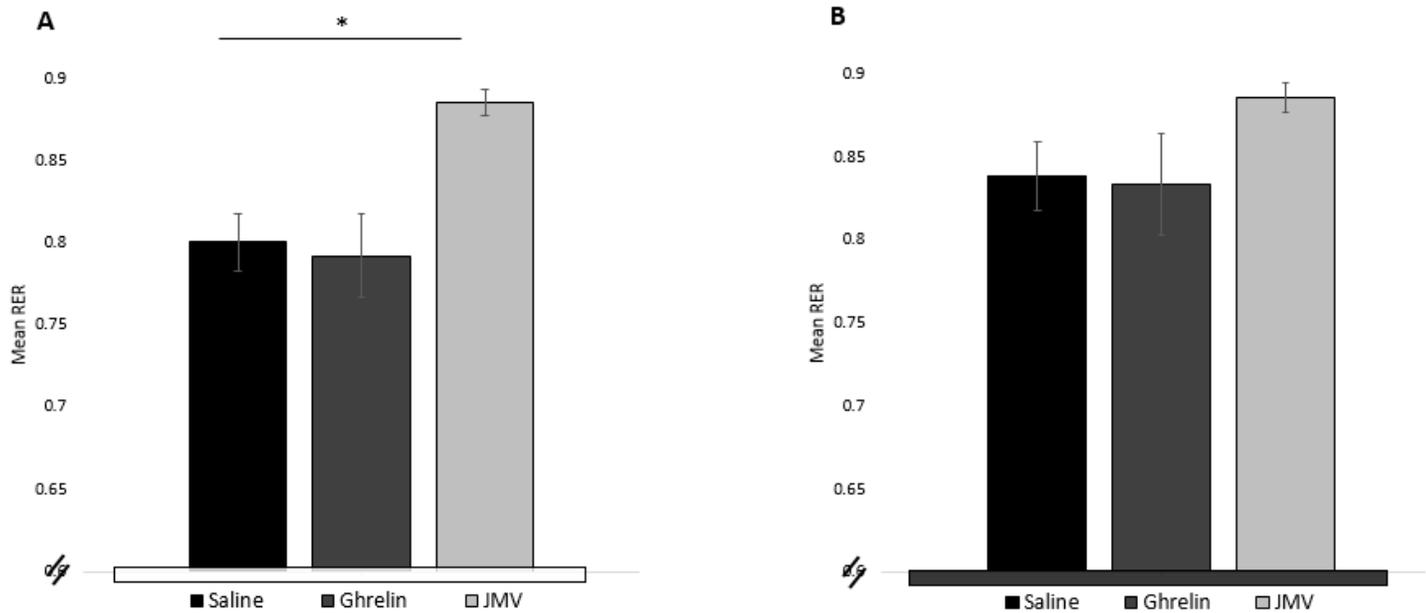


Figure 14A. Mean respiratory exchange ratio during the light phase (+/- SEM). Figure 14B. Mean respiratory exchange ratio during the dark phase (+/- SEM).

Exogenously stimulating the GHSR in the DMH reduces energy expenditure in both the light and dark phase of the light-dark cycle

Energy Expenditure

Individual 30 minute energy expenditure values across a 24 hour period is shown in figure 15. Analysis of these data revealed a significant main effect of drug during the light phase (figure 16A) ($F(2,22)=3.815$, $p<0.05$), partial $\eta^2=0.257$, post-hoc ghrelin vs saline ($p<0.05$), as well as the dark phase (figure 16B) ($F(2,22)=3.578$, $p<0.05$, partial $\eta^2=0.245$, post-hoc ghrelin vs saline ($p<0.05$), JMV2959 vs saline ($p<0.05$). Additionally, energy expenditure changed across time during both the light and dark phase, resulting in a significant main effect of time (light phase; ($F(11.187, 246.117)=5.391$, $p<0.0001$, partial $\eta^2=0.197$, dark phase; ($F(12.203, 268.464)=5.019$, $p<0.0001$, partial $\eta^2=0.186$, but this is primarily due to the expected circadian

pattern of fuel utilization. A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

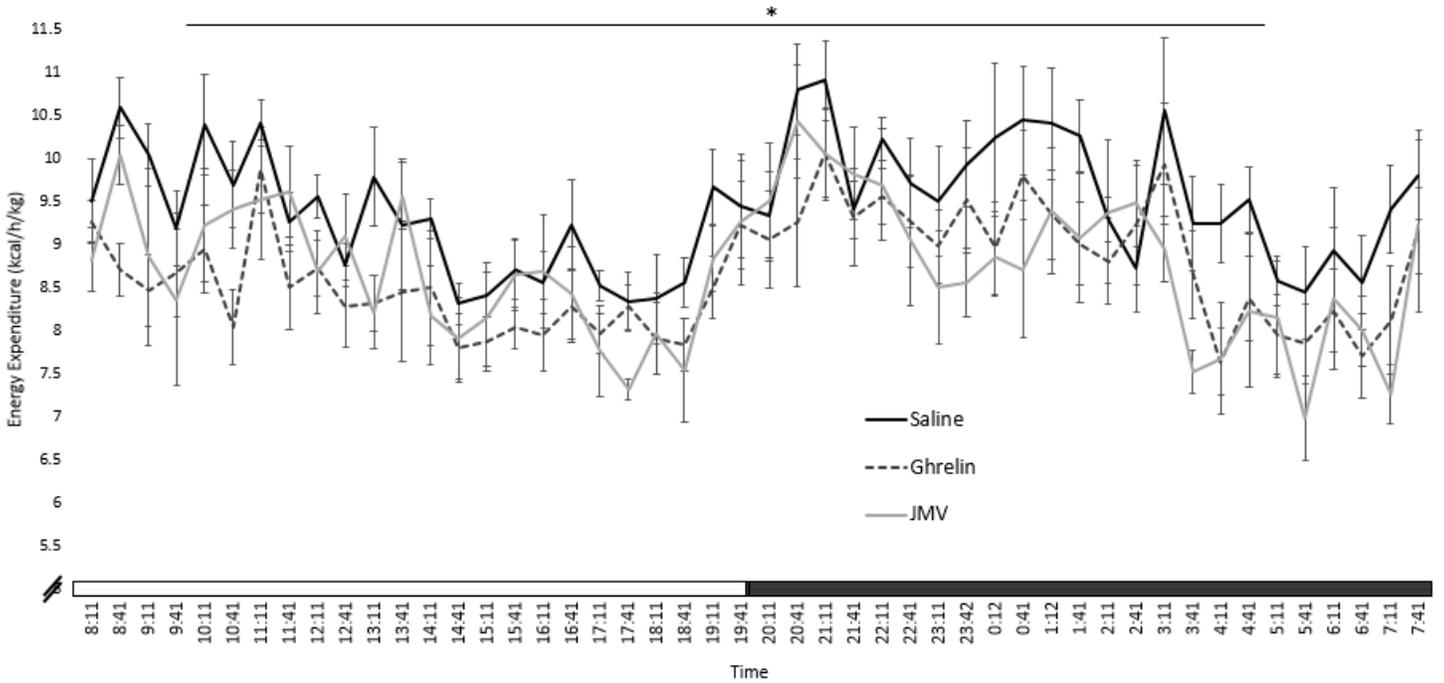


Figure 15. Energy expenditure (kcal/h/kg, +/-SEM) at 30 minute intervals across a 24 hour period. White bar indicates the light phase, black bar indicates dark phase.

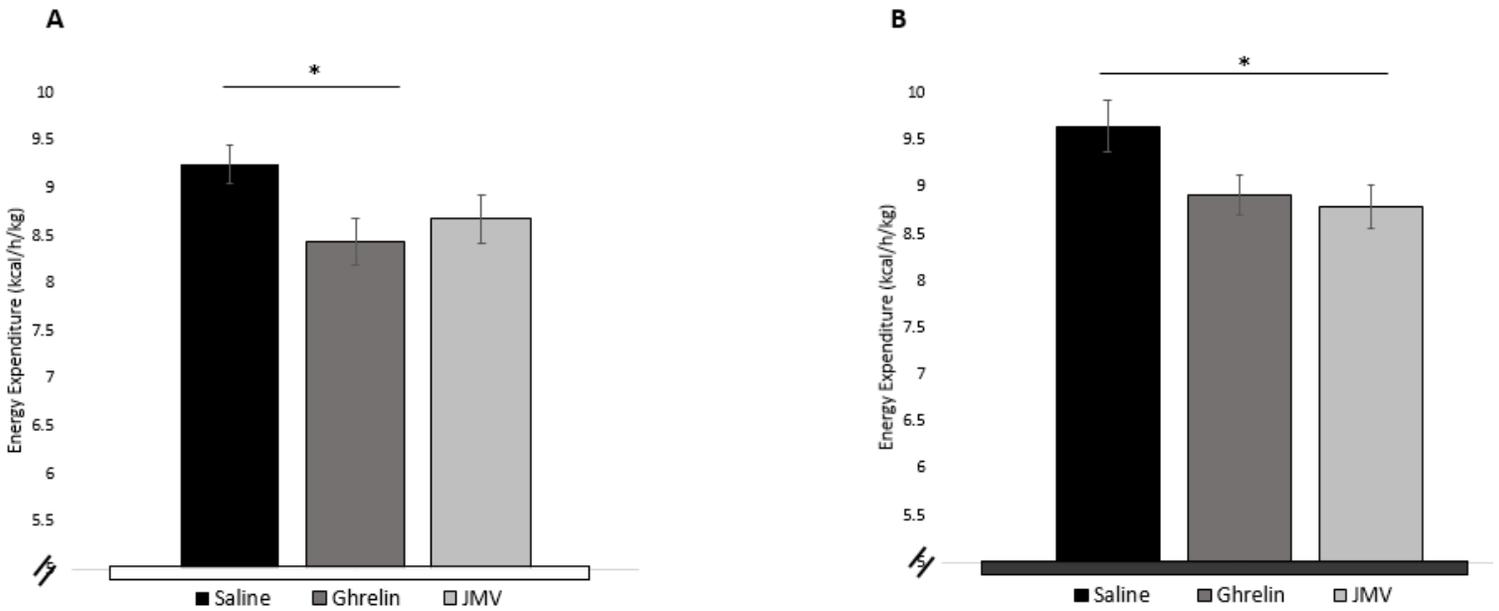


Figure 16A. Mean energy expenditure (kcal/h/kg) during the light phase (+/- SEM). Figure 16B. Mean energy expenditure (kcal/h/kg) during the dark phase (+/- SEM).

Exogenously stimulating the GHSR in the DMH reduces glucose clearance

Glucose tolerance test

Glucose measurements across a 2 hour period following an injection of glucose and area under the curve for glucose clearance are shown in figures 17A and 17B, respectively. The overall repeated measures ANOVA revealed no significant main effect of drug ($F(2,22)=0.737$, $p>0.05$), $\eta^2=0.068$. However, glucose changed across time (figure 17A), resulting in a significant main effect of time ($F(3,66)=379.795$, $p<0.0001$, $\eta^2=0.945$), and the pattern appears to differ between groups, as the group X time interaction approached significance ($F(6,66)=2.127$, $p=0.06$, $\eta^2=0.162$). This appears to be driven by an increase in ghrelin treated animals at 30 minutes post-injection, as supported by a post-hoc at this time point showing ghrelin animals are significantly different from both saline and JMV2959 ($p<0.05$), and by a decrease in JMV2959 treated animals at 15 minutes post-injection, as supported by a post-hoc at this time point showing JMV2959 treated animals are significantly different from both ghrelin and saline treated animals ($p<0.05$). Additionally, there was a significant main effect of drug on the area under the curve (figure 17B) ($F(2,22)=4.554$, $p<0.05$, $\eta^2=0.293$), post-hoc ghrelin vs JMV2959 ($p<0.05$). No significant difference between saline treated animals and either ghrelin or JMV2959 treated animals in area under the curve.

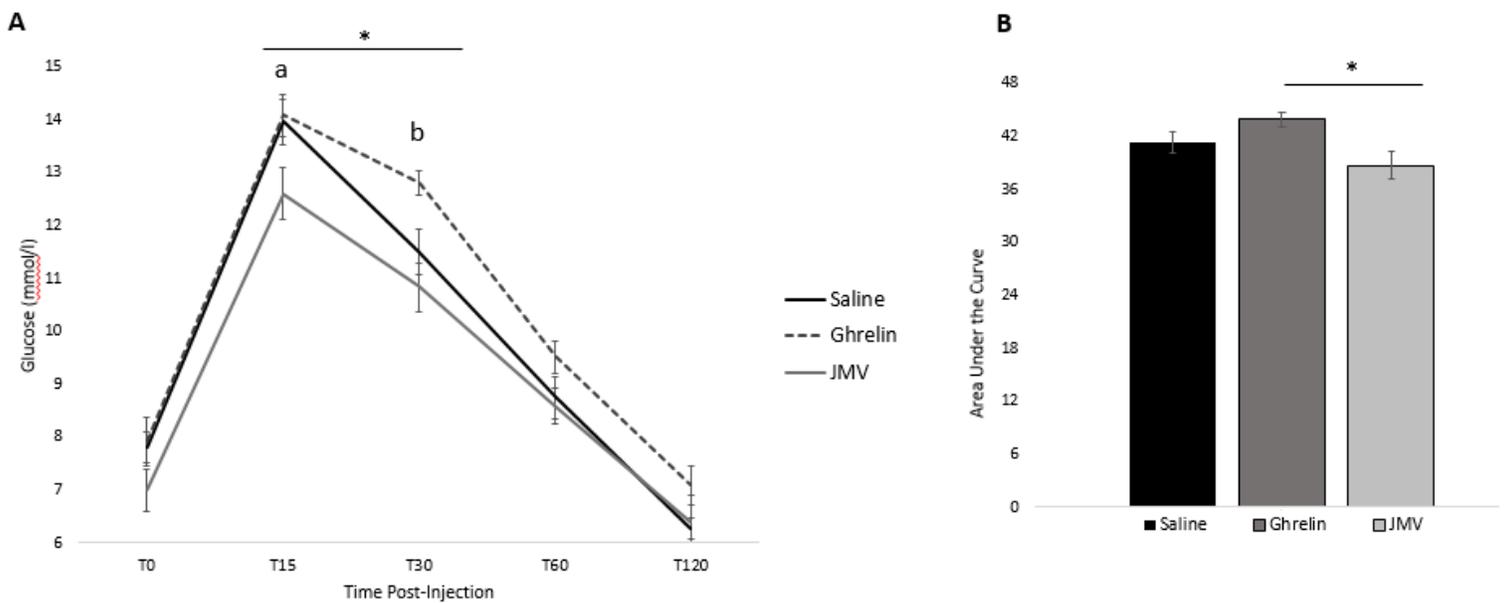


Figure 17A. Glucose levels during the glucose tolerance test (mmol/l) +/-SEM). a=JMV different from ghrelin and saline ($p < 0.05$), b=ghrelin different from JMV and saline ($p < 0.05$). **Figure 17B.** Area under the curve for glucose clearance (+/-SEM).

Exogenously stimulating the GHSR in the DMH increases fat mass

Body composition

Ghrelin treated animals have more fat mass than saline treated animals at the end of the experimental period. A univariate ANOVA on the ratio of fat mass to total body weight (figure 18A), identified a main effect of drug that approached significance ($F(2,21)=3.064$, $p=0.068$, partial $\eta^2=0.226$). However, given our hypothesis and the large body of literature showing that ghrelin promotes adiposity, we did a planned comparison between ghrelin and saline, and found that ghrelin treated animals differ significantly from saline treated animals. A univariate ANOVA on the ratio of fat mass to lean mass (figure 18B) similarly showed a main effect of drug approached significance ($F(2,21)=3.077$, $p=0.067$, partial $\eta^2=0.227$), and a planned post-hoc comparison ghrelin vs saline ($p < 0.05$).

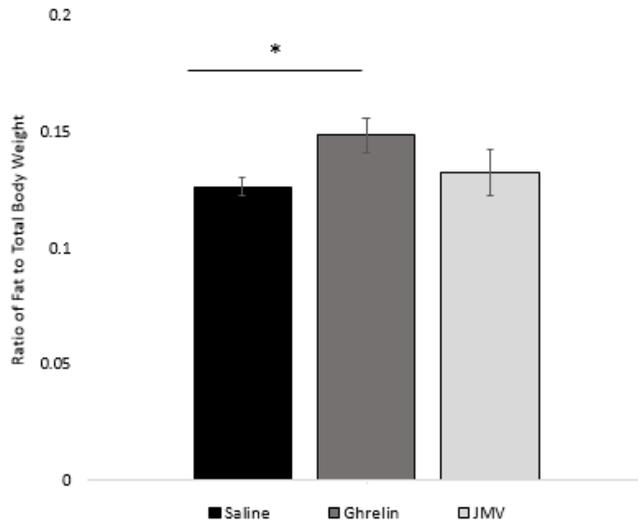
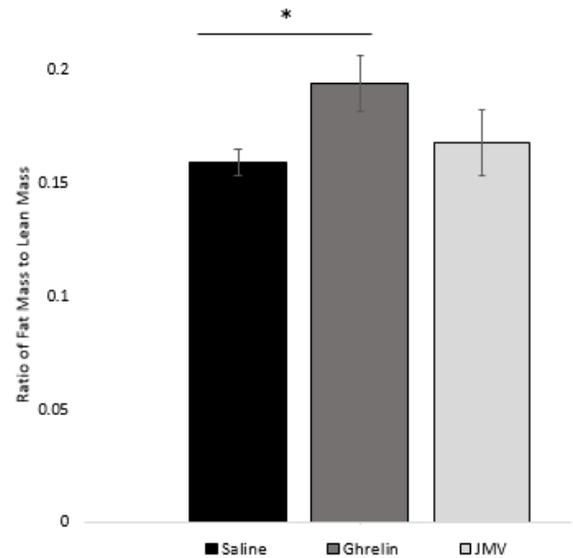
A**B**

Figure 18A. Ratio of fat mass to body weight (+/- SEM). Figure 18B. Ratio of fat mass to lean mass (+/- SEM)

Manipulating ghrelin receptor signaling in the DMH does not affect anticipation to scheduled high-fat diet exposure

Anticipatory Locomotor Activity

Mean locomotor anticipatory activity is shown in figure 19. Analysis of these data revealed no significant main effect of drug ($F(2,22)=0.621$, $p>0.05$), partial $\eta^2=0.053$. Additionally, locomotor activity changed across time ($F(10,220)=3.509$, $p<0.001$, partial $\eta^2=0.138$, however a similar pattern was seen in all groups, and the group X time interaction effect was not significant. Additionally, anticipatory activity is typically measured as increases in locomotor activity 2-3 hours prior to a scheduled meal, compared activity at or after the presentation of the meal. Thus, we compared each of the 30 minute time points prior to the scheduled high fat diet (H), to activity at H, and found that activity was not significantly elevated at any time point compared to H, indicating they did not anticipate the meal.

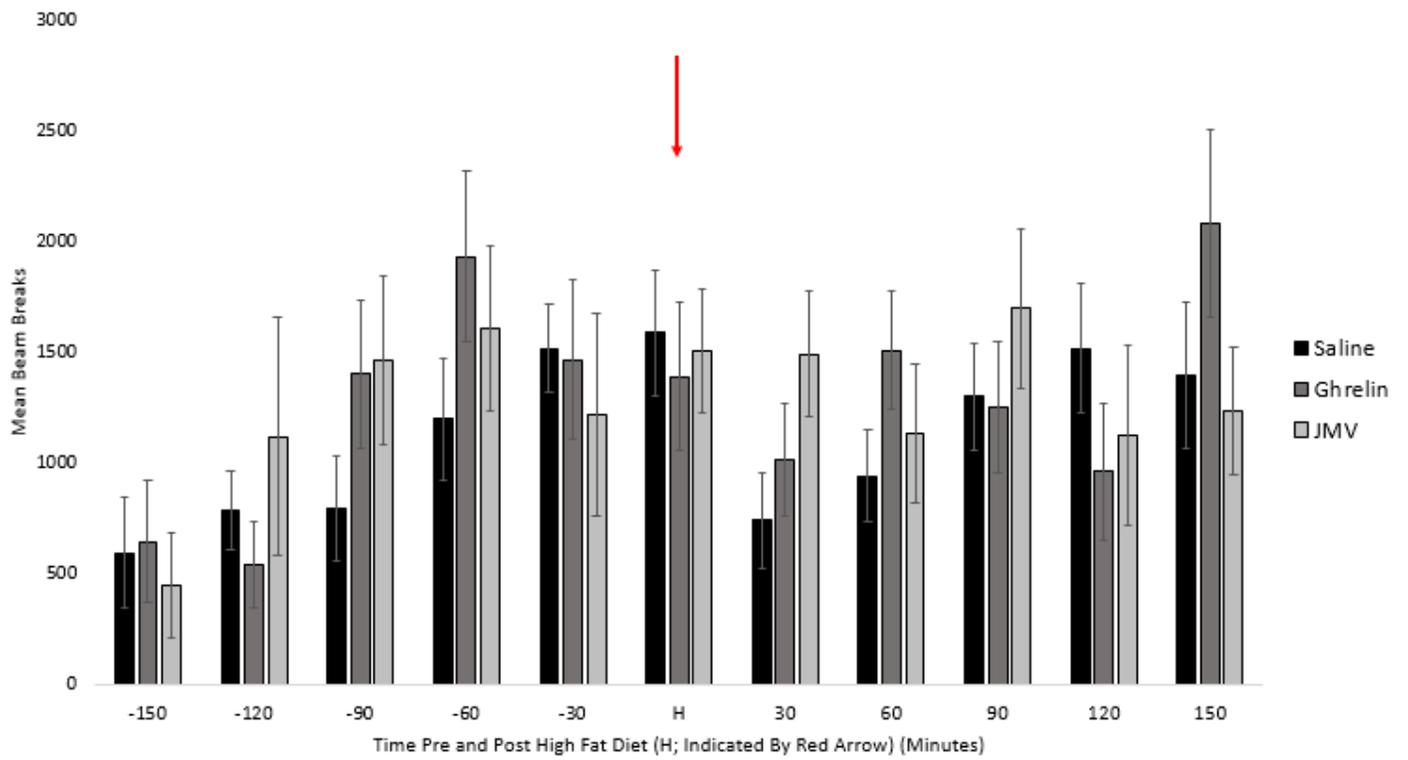


Figure 19. Mean anticipatory locomotor activity (beam breaks; +/- SEM) to scheduled high-fat diet exposure.

Experiment 3: Effects of manipulating ghrelin receptor signaling within the PMV

To determine whether GHSR signaling in the PMV is important for energy balance or contributes to anticipation of a scheduled treat, and whether the effects of manipulating the GHSR in the DMH are region-specific, saline, ghrelin, or DLYS-GHRP6 were infused into the PMV for 18 days. 3 animals did not recover from surgery or were sacrificed and were thus removed from all analyses. Further, 7 animals were removed due to incorrect cannula placements. Thus, 20 animals were included in the final analysis, with the following group numbers: saline (n=6), ghrelin (n=8), DLYS-GHRP6 (n=6).

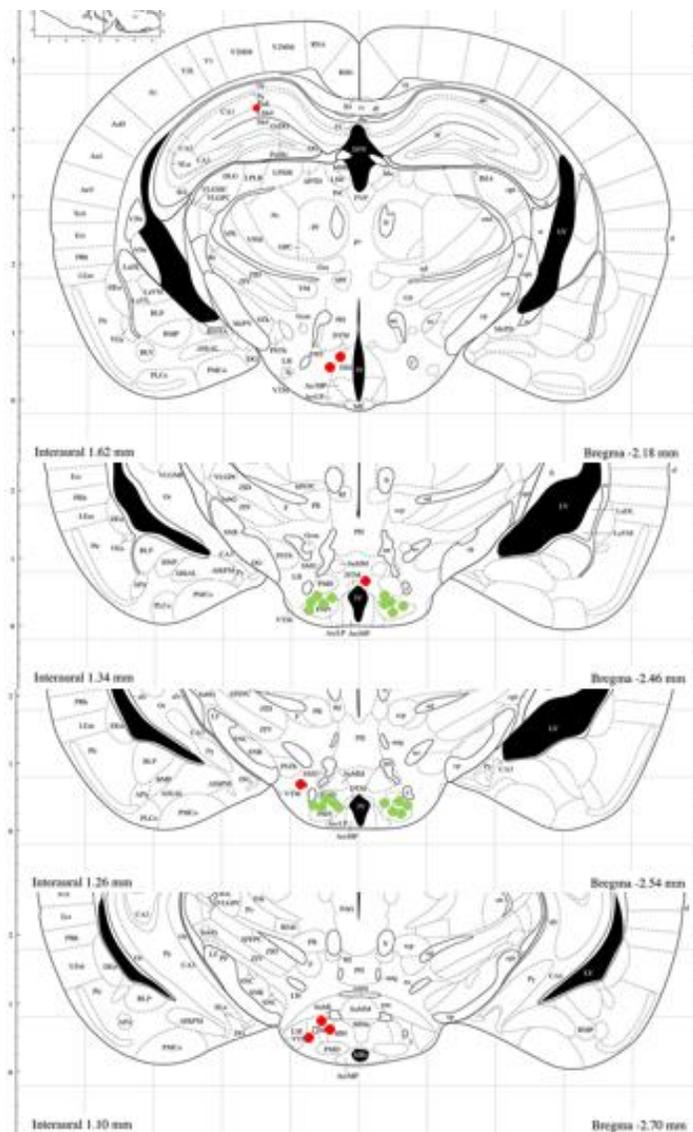


Figure G. Summary of cannula placements for experiment 3. The red dots represent placements falling outside the PMV and were therefore excluded from all analyses. The green dots represent those that fall within or just above the PMV and were therefore included in the analysis.

Manipulating ghrelin receptor signaling in the PMV does not affect body weight or caloric intake

Body Weight Change

Daily change in body weight across the 16 days of the experiment is shown in figure 20. The overall repeated measures ANOVA revealed no significant main effect of drug ($F(2,17)=1.446, p>0.05$), partial $\eta^2=0.145$. Additionally, body weight increased across time, and these increases are suppressed by the overnight fast imposed on the animals for the GTT resulting in a significant main effect of time ($F(8.54,145.186)=32.011, p<0.0001$, partial $\eta^2=0.653$). A similar pattern was seen in all groups, and the group X time interaction effect was not significant.

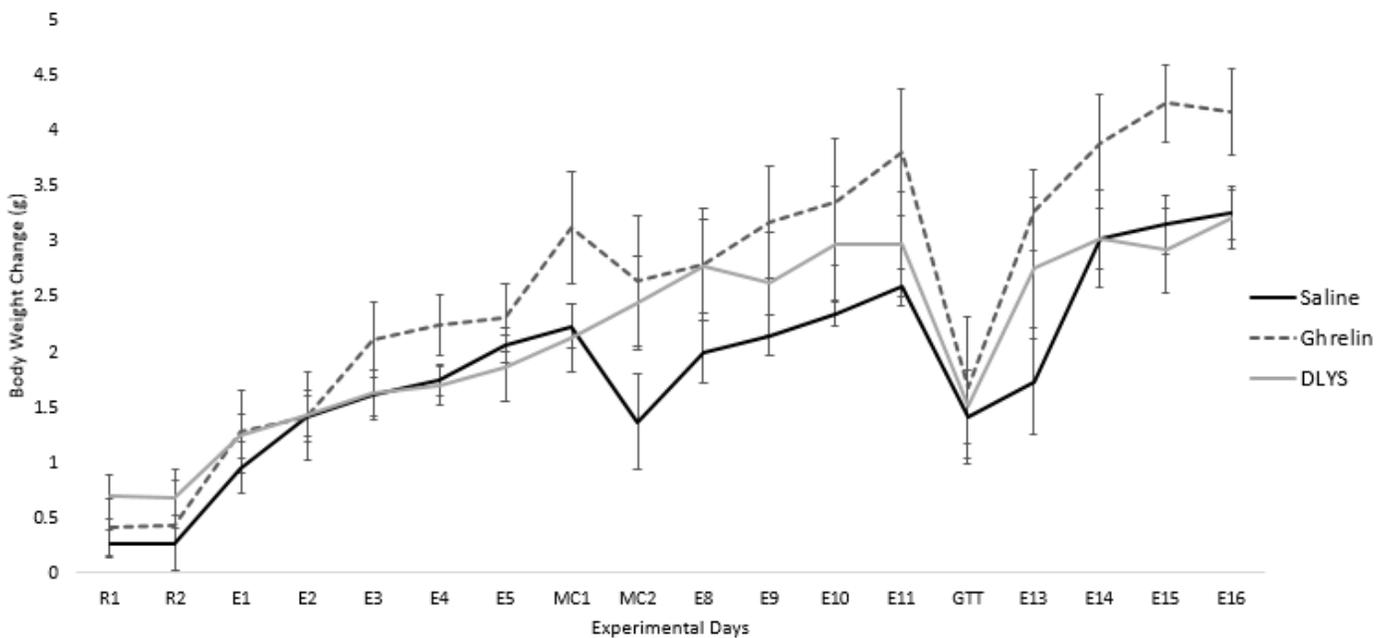


Figure 20. Mean daily body weight change (g) from day 1 to day 16 of the experimental period.

Chow Intake Change

Daily change in chow intake across the 16 days of the experiment is shown in figure 21. The overall repeated measures ANOVA revealed no significant main effect of drug ($F(2,17)=0.174$, $p>0.05$), partial $\eta^2=0.020$. Additionally, chow intake changed across time, significant main effect of time ($F(13.408, 227.935)=35.561$, $p<0.001$, partial $\eta^2=0.677$). However, this appears to be primarily due to the lack of food intake during the overnight fast imposed on the mice for the GTT paradigm and the post-fast rise in food intake. A similar pattern was seen in all groups, and the group X time interaction effect was not significant

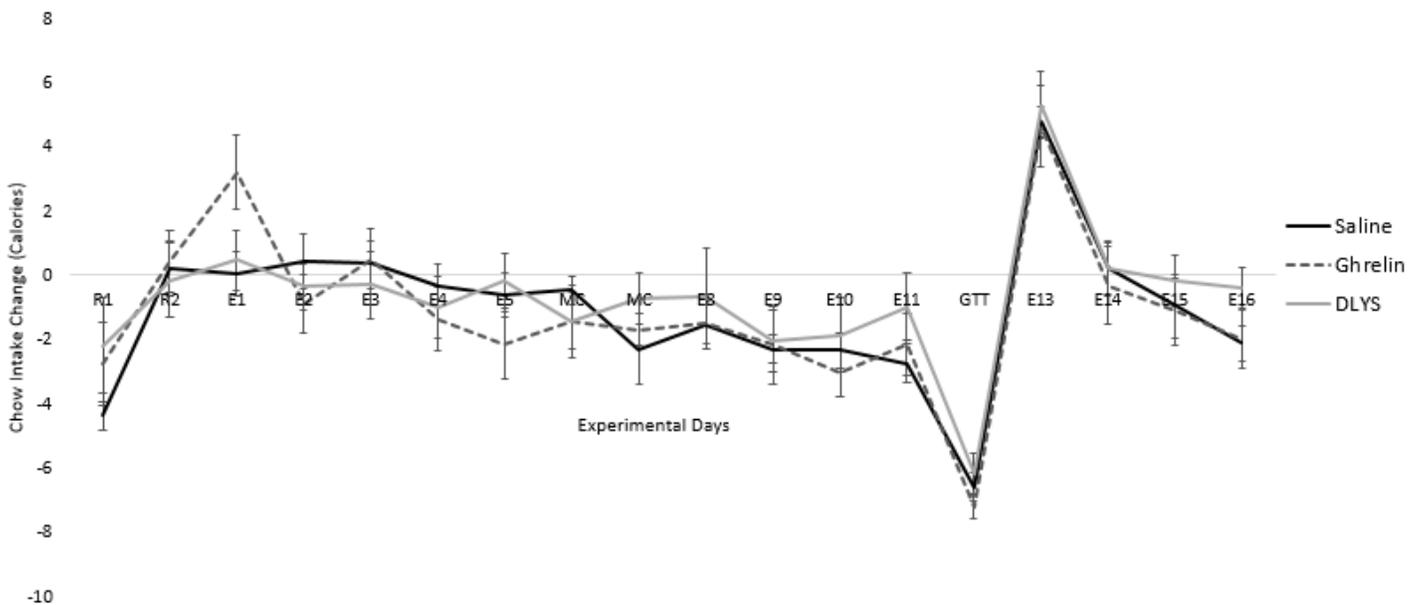


Figure 21. Mean daily chow intake change (calories) from day 1 to day 16 of the experimental period.

High Fat Diet Intake Change

Daily change in high fat diet intake across the 16 days of the experiment is shown in figure 22. The overall repeated measures ANOVA revealed no significant main effect of drug ($F(2,17)=1.897$, $p>0.05$), partial $\eta^2=0.182$. Additionally, high fat diet intake changed across time,

significant main effect of time ($F(12.394, 567.885)=34.501, p<0.001, \text{partial } \eta^2=0.67$). However, this appears to be primarily due to the lack of food intake during the overnight fast imposed on the mice for the GTT paradigm and the post-fast rise in food intake. A similar pattern was seen in all groups, and the group X time interaction effect was not significant

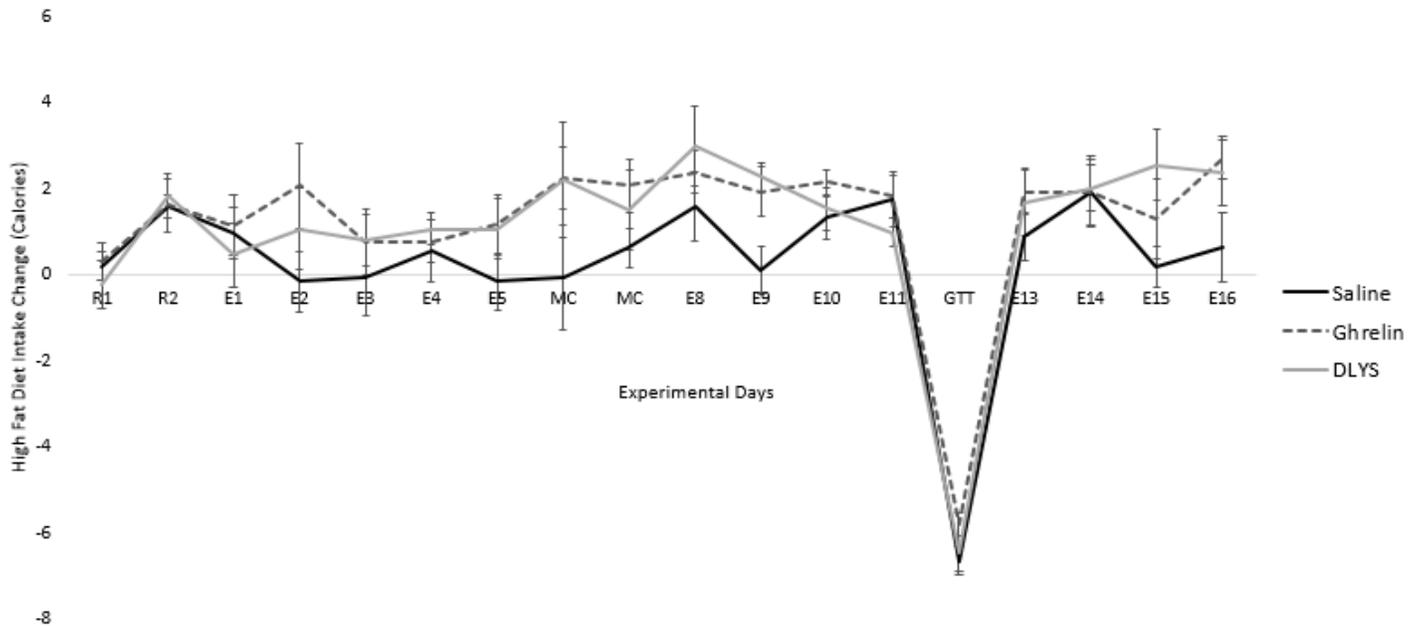


Figure 22. Mean daily high fat diet intake change (calories) from day 1 to day 16 of the experimental period.

Total Caloric Intake Change

Daily change in total caloric intake across the 16 days of the experiment is shown in figure 23. The overall repeated measures ANOVA revealed no significant main effect of drug ($F(2,17)=1.378, p>0.05$), partial $\eta^2=0.14$. Additionally, total caloric intake changed across time, significant main effect of time ($F(15, 255)=60.614, p<0.001, \text{partial } \eta^2=0.781$). However, this appears to be primarily due to the lack of food intake during the overnight fast imposed on the mice for the GTT paradigm and the post-fast rise in food intake. A similar pattern was seen in all groups, and the group X time interaction effect was not significant

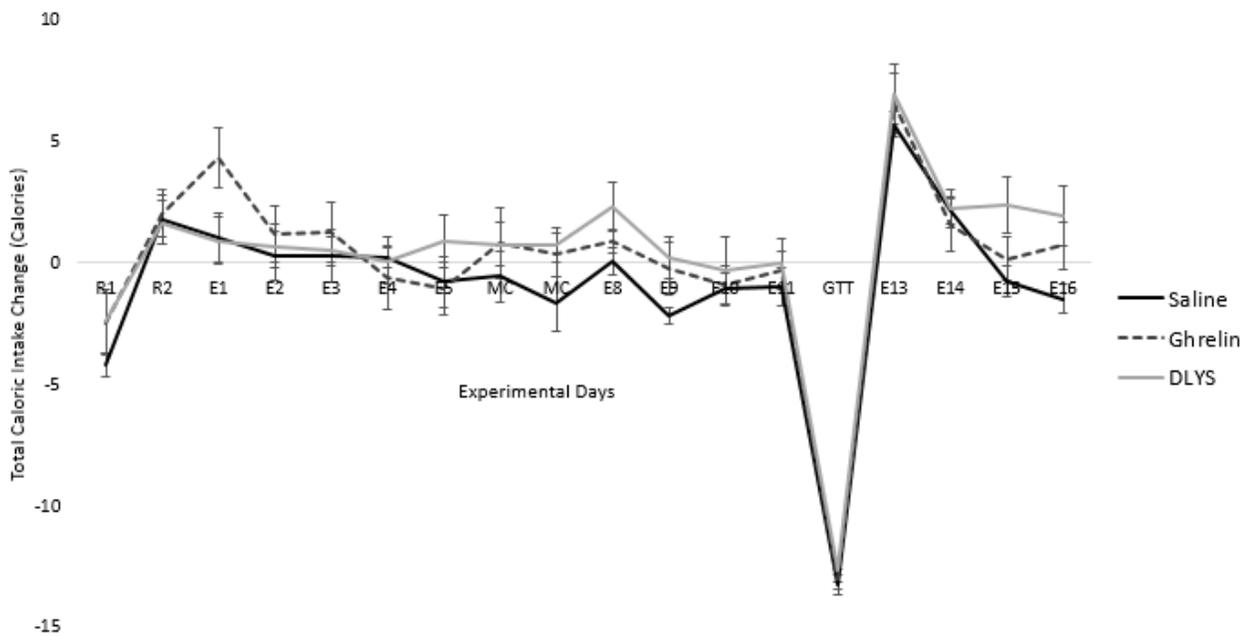


Figure 23. Mean daily total caloric intake change (calories) from day 1 to day 16 of the experimental period.

Manipulation of ghrelin receptor signaling in the PMV promotes carbohydrate metabolism during both the light and dark phase of the light-dark cycle

Respiratory exchange ratio

Individual 30 minute RER values across a 24 hour period is shown in figure 24. Analysis of these data revealed a significant main effect of drug during the light phase (figure 25A) ($F(2,17)=5.399$, $p<0.05$), partial $\eta^2=0.388$, post-hoc DLYS-GHRP6 vs saline ($p<0.05$), ghrelin vs saline ($p<0.05$), as well as the dark phase (figure 25B) ($F(2,17)=4.043$, $p<0.05$, partial $\eta^2=0.322$, post-hoc DLYS-GHRP6 vs saline ($p<0.05$), ghrelin vs saline ($p<0.05$). Additionally, RER changed across time during both the light and dark phase, resulting in a significant main effect of time (light phase; ($F(17.031, 289.528)=7.161$, $p<0.001$, partial $\eta^2=0.296$, dark phase; ($F(12.131, 206.232)=2.804$, $p<0.001$, partial $\eta^2=0.142$, but this is primarily due to the expected circadian pattern of fuel utilization. This pattern differed by group, and the group X time interaction effect was significant during the light phase ($F(34.062, 289.528)=1.828$, $p<0.01$, partial $\eta^2=0.177$. A

similar pattern was seen in all groups during the dark phase, and the group X time interaction effect was not significant.

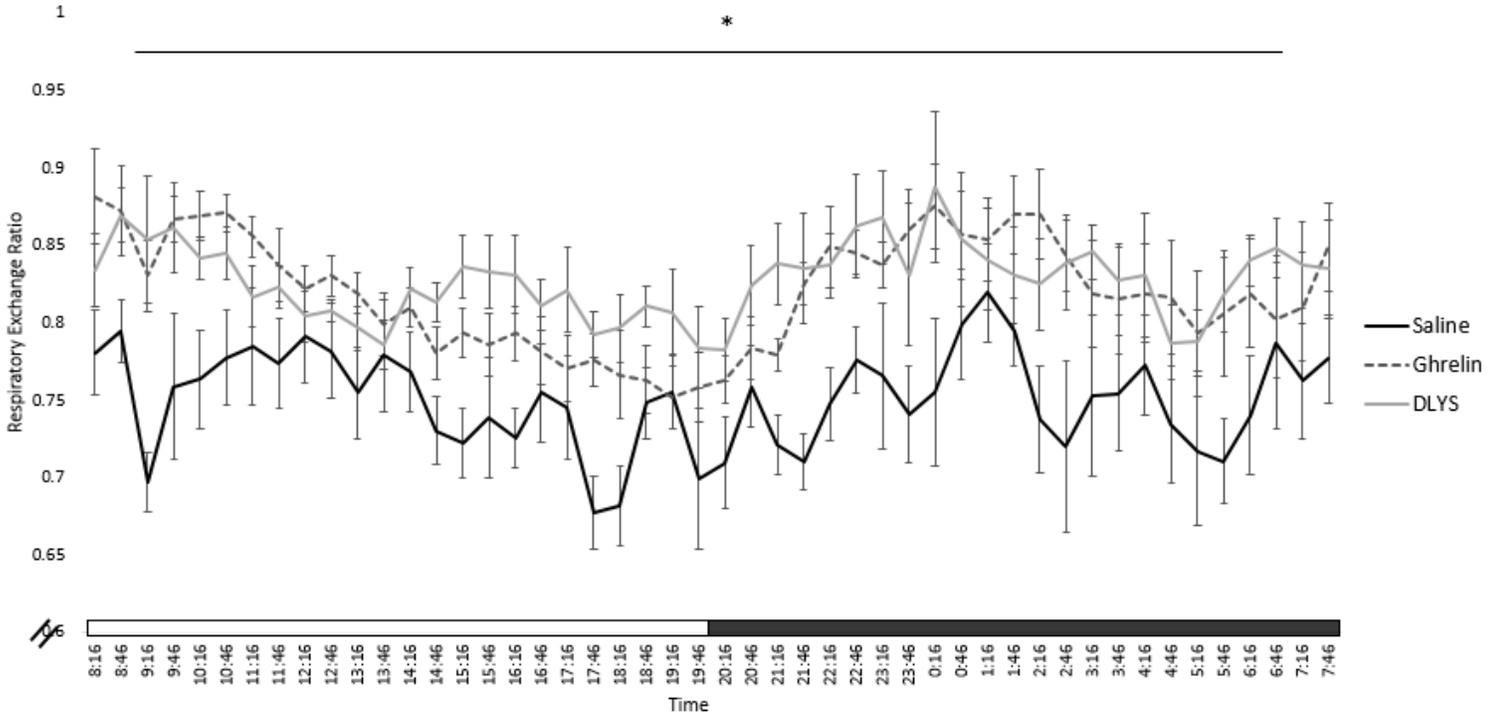


Figure 24. Mean respiratory exchange ratio at 30 minute intervals across a 24 hour period. White bar indicates light phase, black bar indicates dark phase.

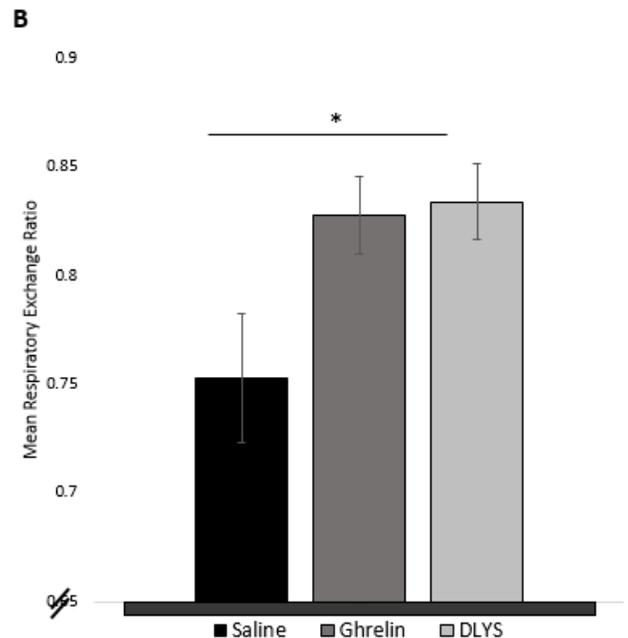
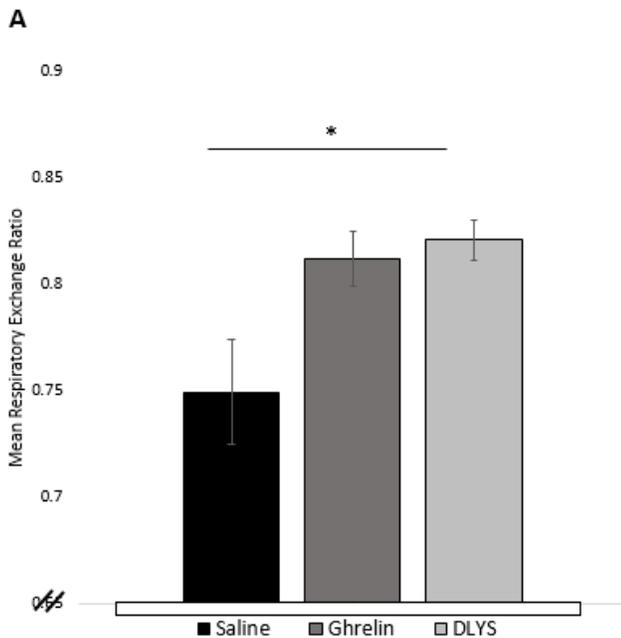


Figure 25A. Mean respiratory exchange ratio during the light phase (+/- SEM). Figure 25B. Mean respiratory exchange ratio during the dark phase (+/- SEM).

Exogenous stimulation of the GHSR in the PMV reduces glucose clearance

Glucose tolerance test

Glucose measurements across a 2 hour period following an injection of glucose and area under the curve for glucose clearance is shown in figures 26A and 26B, respectively. The overall repeated measures ANOVA revealed a significant main effect of drug ($F(2,16)=5.824$, $p<0.05$), $\eta^2=0.421$, post-hoc ghrelin vs saline ($p<0.01$). Additionally, as expected, glucose changed across time (figure 26A), resulting in a significant main effect of time ($F(4,64)=101.939$, $p<0.0001$, $\eta^2=0.864$, however the pattern was similar among groups, and the group X time interaction was not significant. Ghrelin treated animals display significantly higher glucose than saline treated animals at 30 minutes and 60 minutes post-injection (post-hoc ghrelin vs saline, $p<0.05$). Additionally, there was a significant main effect of drug on the area under the curve (figure 26B) ($F(2,16)=5.131$, $p<0.05$, $\eta^2=0.391$), post-hoc ghrelin vs saline ($p<0.01$).

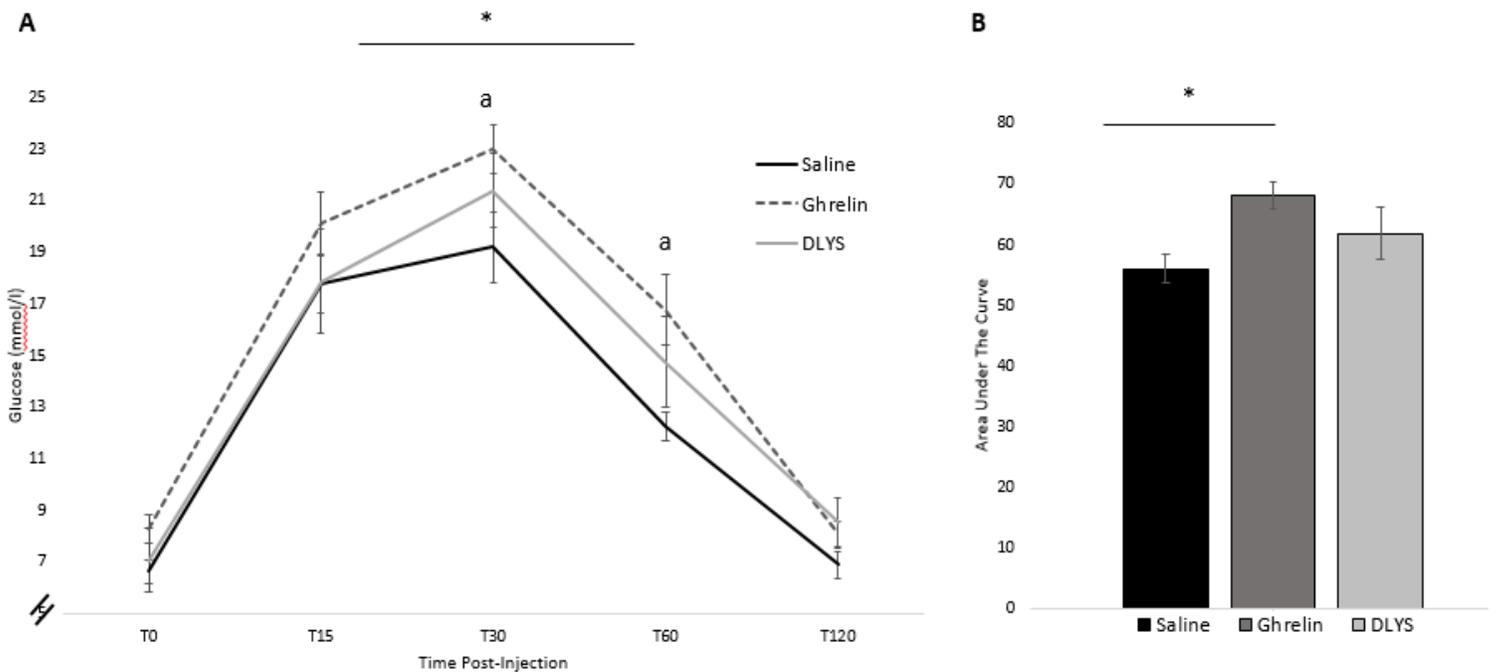


Figure 26A. Glucose levels during the glucose tolerance test (mmol/l +/-SEM). a=Ghrelin different from saline ($p<0.05$). Figure 26B. Area under the curve for glucose clearance (+/-SEM).

Manipulating ghrelin receptor signaling in the PMV does not affect body composition

Mean ratio of fat mass to total body weight and ratio of fat mass to lean mass can be found in figure 27A and 27B, respectively. There were no differences between the groups in either ratio (fat mass to body weight; $F(2,16)=0.283$, $p>0.05$, partial $\eta^2=0.034$, fat mass to lean mass; $F(2,16)=0.368$, $p>0.05$, partial $\eta^2=0.044$).

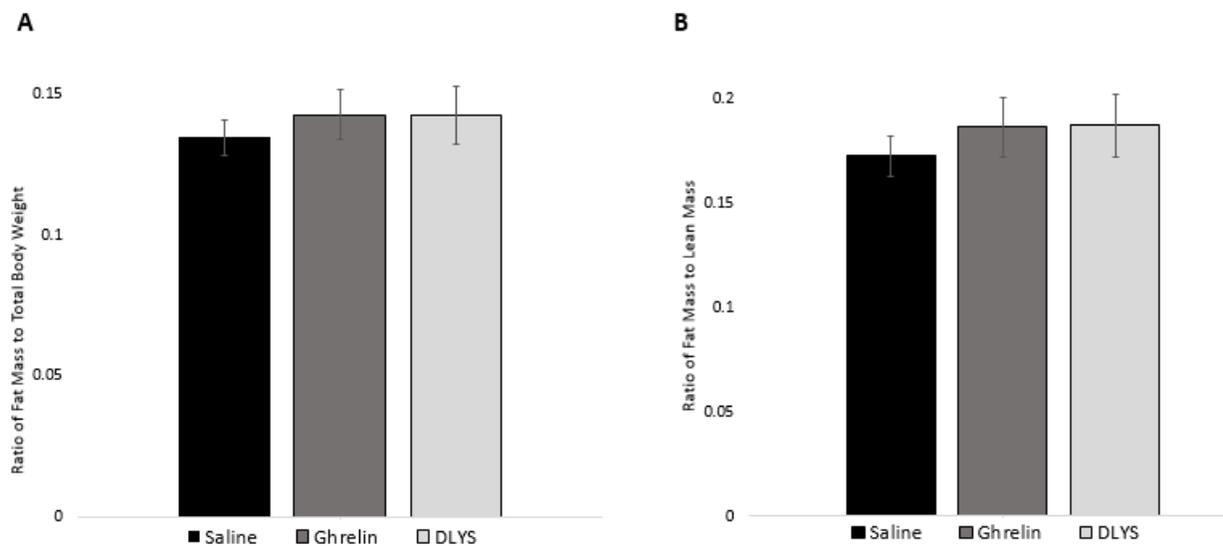


Figure 27A. Ratio of fat mass to body weight (+/- SEM). Figure 27B. Ratio of fat mass to lean mass (+/- SEM)

Manipulating ghrelin receptor signaling in the PMV does not affect anticipation to scheduled high-fat diet exposure

Anticipatory Locomotor Activity

Mean locomotor anticipatory activity is shown in figure 28. Analysis of these data revealed no significant main effect of drug ($F(2,17)=0.6$, $p>0.05$), partial $\eta^2=0.066$. Additionally, locomotor activity changed across time ($F(8.458,143.793)=6.713$, $p<0.001$, partial $\eta^2=0.283$, however a similar pattern was seen in all groups, and the group X time interaction effect was not

significant. Additionally, anticipatory activity is typically measured as increases in locomotor activity 2-3 hours prior to a scheduled meal, compared activity at or after the presentation of the meal. Thus, we compared each of the 30 minute time points prior to the scheduled high fat diet (H), to activity at H, and found that activity was not significantly elevated at any time point compared to H, indicating they did not anticipate the meal.

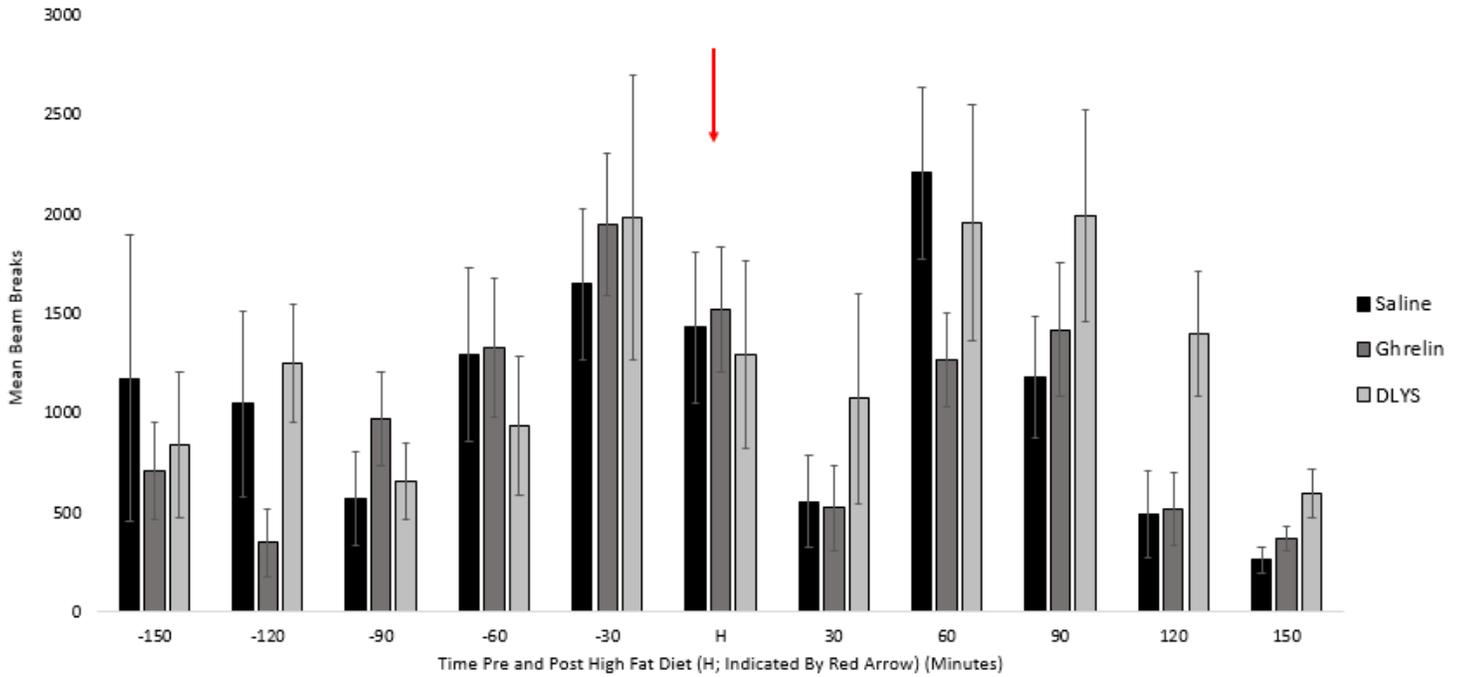


Figure 28. Mean anticipatory locomotor activity (beam breaks; +/- SEM) to scheduled high-fat diet exposure.

Discussion

The studies described in this thesis were designed to test the hypothesis that GHSR signalling in the dorsomedial hypothalamus is important for energy balance in mice. To do this, pharmacological manipulations to alter ghrelin binding to the receptor within the DMH and the PMV were employed. The DMH is an orexigenic nucleus that is important for modulating energy balance via its effects not only on food intake but also on thermoregulation, as well as food-entrainable circadian rhythms. The PMV is an integrative area important for coordinating the timing of reproduction with food availability, but has no known role in energy balance. Thus, we hypothesized that chronic administration of ghrelin in the DMH but not in the PMV would increase food intake, body weight, and anticipation to a scheduled meal. Chronic administration of a GHSR antagonist in the DMH, on the other hand, was expected to attenuate food intake, body weight, and anticipation to a scheduled. Further, since lesions of the PMV point to it being an important glucoregulatory area, we hypothesized that chronic administration of ghrelin into both the DMH and the PMV would slow glucose clearance, while administration of a GHSR antagonist into either region would facilitate its clearance.

Data from our first experiment allowed us to choose an appropriate dose of the GHSR antagonist JMV2959 for our next 2 experiments. While this antagonist has become increasingly popular, no studies have been published using chronic administration of this compound into hypothalamic nuclei in mice. Thus, we ran a pilot study where we chronically administered a low dose of JMV2959 (2 $\mu\text{g}/\text{day}$), or a high dose (20 $\mu\text{g}/\text{day}$) into the DMH or the PMV of adult male mice. We found that the lower dose was more effective than the high dose at attenuating total caloric intake (table 1), and hence used it in experiment 2.

Contrary to our hypothesis, in experiment 2, we found no effect of chronic ghrelin infusion on caloric intake. However, as expected, infusions of JMV959 into the DMH attenuated total caloric intake compared to both saline and ghrelin treated animals, primarily by reducing calories from chow, but not from the high fat diet. However, because the metabolic chambers and overnight fast imposed on the animals for the glucose tolerance test are both stressful manipulations, we separated the experimental period into three phases, and investigated the change in caloric intake and body weight within each phase. This allowed use to delineate not only the effects of the metabolic chambers and glucose tolerance test on our measurements, but also how our drug manipulation continued to effect body weight and food intake. We found that the attenuation in caloric intake of JMV2959 treated animals began at the onset of drug infusion and persisted at a similar level for the remainder of the experiment, as there were no differences in caloric intake during the second and third phases with respect to the prior phase. This also indicates that neither the stress of the metabolic chambers nor the overnight fast altered the efficacy of JMV2959 in suppressing caloric intake. These results are consistent with the notion that ghrelin signalling in the DMH modulates food intake and that this effect is diet specific.

The decrease in caloric intake seen in JMV2959 treated mice contrasts with the findings of Merkenstein et al., 2014, who showed that viral knockdown of the GHSR in the DMH of rats did not affect food intake. However, there are a number of differences between the present experiment and that of Merkenstein et al., 2014. First, our model used mice rather than rats. Second, the animals in the Merkenstein et al., 2014 experiment were given access to a running wheel, and were not given a choice of diet, while our animals were not given access to a running wheel, and were provided with a diet choice. Finally, it may be that since viral knockdown does not completely deplete the region of the GHSR, our pharmacological approach may be a more

radical method. Which of these methodological differences accounts for the discrepancies between the findings of the studies is currently unclear.

A number of studies have demonstrated that ghrelin increases preference for diets high in carbohydrate over fat-rich diets (Schele, Bake, Rabasa, and Dickson, 2016). Indeed, disruption of GHSR1a signaling in the PVN of mice increases high fat diet intake of both stressed and non-stressed animals (Patterson, Parno, Isaacs, and Abizaid, 2013). Thus, the current finding that the effect of antagonist administration was seen primarily in chow intake suggests that ghrelin signalling in the DMH may play a role in this food preference. Although Yang et al., 2009 found that DMH NPY is important for meal size, and thus caloric intake, to our knowledge this is the first study to suggest that the DMH plays an important role in macronutrient selection, although the lack of effects of ghrelin infusion itself suggests that these results are preliminary. However, while we are unsure of why we did not see an increase in food intake in ghrelin-treated animals, it is possible that it is rapid desensitization of the receptor accounting for this. Indeed, in Chinese hamster ovary cells expressing the human GHSR, the GH response to two consecutive ghrelin pulses is significantly reduced when the pulses are separated by an interval of one hour, whereas an interval of 2.5 hours or longer retains the GH response (Yin, Li, and Zhang, 2014). The authors also report that this desensitization is a result of receptor internalization, an effect that depends on constitutive activity of the receptor (Yin, Li, and Zhang, 2014). Additionally, the receptor shows a slow return to normal cell surface expression, return to 100% recovery only 6 hours after agonist removal (Yin, Li, and Zhang, 2014). While ghrelin is rapidly broken down in both rat and human serum (half-life of 27 minutes and 236 minutes, respectively), it is possible that continuous chronic infusion of ghrelin induces desensitization of the receptor, mitigating the effects of ghrelin on food intake.

As expected, mice treated with ghrelin had gained more fat mass by the end of the experiment than those treated with either JMV2959 or saline. The ability of ghrelin to increase weight gain was, however, limited to phases 1 and 2 of the experiment. The increased weight gain of ghrelin treated mice is presumably a result of a decrease in energy expenditure, since food intake and locomotor activity were unaffected. To assess this, we analyzed energy expenditure with lean mass as a covariate, a method recommended by many mouse metabolism researchers (Tschop et al., 2011; Butler and Kozak, 2010). Consistent with previous research by Wren et al., 2001, ghrelin treated animals had lower energy expenditure than saline treated animals, but in the current experiment this was only seen during the dark phase (see appendix). Counterintuitively, JMV2959 mice also showed a reduction in energy expenditure relative to saline treated animals that was seen in both the light and dark phase of the light-dark cycle. In neither ghrelin nor the JMV2959 groups was there an effect of treatment on locomotor behaviour. This reduction in energy expenditure probably contributed to the fact that JMV2959 treated mice gained as much weight across the experiment as saline treated animals in spite of their reduction in caloric intake.

While it is surprising that both JMV2959 treated animals and ghrelin treated animals display reduced energy expenditure, even when accounting for changes in total caloric intake and lean mass, it is possible that it is cyclic occupancy of the ghrelin receptor in the DMH that is important for modulating energy expenditure, since both infusions of ghrelin and JMV2959 eliminate the endogenous daily rhythm in ghrelin binding. Furthermore, carbohydrate metabolism is significantly elevated in JMV2959 treated animals relative to ghrelin and saline treated animals, as reflected by the increase in RER during the light phase whereas ghrelin itself had no effect. This is also a surprising finding because ghrelin typically promotes the use of

carbohydrates as a fuel source, which are easily metabolized and a quicker fuel source than fats (Abtahi, Mirza, Howell, and Currie, 2017). However, it may be that it is an agonistic effect of JMV2959 causing the reduction in energy expenditure and increase in carbohydrate oxidation. Indeed, M'Kadmi et al., 2015 used in vitro assays to show that JMV2959 only acts as a partial antagonist. While JMV2959 is an antagonist to the ghrelin-induced MAPK/ERK pathway (M'Kadmi et al., 2015), it is a partial agonist to the IP₃ pathway (M'Kadmi et al., 2015). M'Kadmi et al., 2015 showed that the G_q pathway, which stimulates PLC production, is agonized by JMV2959 (M'Kadmi et al., 2015), which leads to the production of intracellular calcium. Therefore, while JMV2959 may be acting as a traditional antagonist to the GHSR and attenuating food intake through some ghrelin induced pathways and physiological functions, it may be acting as an agonist in other pathways, particular those that modulate energy expenditure.

There are three components contributing to energy expenditure rates; basal metabolic rate, adaptive thermogenesis, and physical activity (Munzberg et al., 2015). Since basal metabolic rate has limited regulation, and physical activity is not affected in these animals, it is therefore likely that the decrease in energy expenditure is through a decrease in adaptive thermogenesis. It is known that the DMH plays an important role in modulating BAT thermogenesis, and stimulating neurons in the DMH increases sympathetic nerve activity to BAT (DiMicco et al., 2007). Thus, it is possible that endogenous ghrelin binding in the DMH either activates or disinhibits DMH neurons, thereby increasing BAT activity, and subsequently energy expenditure.

The DMH has also been implicated in glucose homeostasis, specifically it has been shown that knockdown of DMH NPY improves glucose homeostasis, while NPY overexpression limits glucose clearance (Li et al., 2016). JMV2959 treated animals in our experiment display

improved glucose clearance in the glucose tolerance test compared to ghrelin treated mice, as shown by a significantly lower area under the curve for glucose clearance, suggesting that DMH GHSR signaling is important for peripheral insulin functioning. While we did not investigate the mechanisms by which we are affecting peripheral glucose, it is possible that we are directly or indirectly affecting DMH NPY. It is well established that ghrelin directly regulates NPY activity and expression in the ARC and it is possible that the effects of ghrelin on glucose clearance in the DMH also involve changes in NPY signaling. Specifically, blocking GHSR signaling may be reducing NPY expression or activity, thereby improving glucose clearance.

In addition to testing whether manipulation of ghrelin receptor activity in the DMH affects food intake, weight gain, fuel metabolism, and energy expenditure, we also wanted to test whether these manipulations alter anticipatory behaviour prior to scheduled high fat diet exposure. We found that not only did manipulation of the receptor have no affect on anticipation, but that none of the groups display an increase in activity in anticipation of the scheduled high fat diet. This may occur for many reasons. First, it is not clear whether high fat diet is sufficiently rewarding to induce anticipatory behaviour, particularly in mice. Food anticipatory activity is typically measured in animals that are placed on a restricted feeding paradigm, and then presented with a scheduled exposure to high fat diet. Our animals, however, are given *ad libitum* access to standard laboratory chow. Circadian experts often use *ad libitum* animals exposed to a scheduled treat known for its rewarding properties, such as cookie dough, as a model of hedonic feeding. However, there is no evidence to suggest that mice on this model of feeding with the replacement of cookie dough with high fat diet will not only find the high fat diet rewarding, but will also anticipate its presence. Thus, our experiment demonstrates that scheduled exposure to a high fat diet does not induce anticipatory activity in free feeding mice. However, whether

manipulations to ghrelin receptor activity in the DMH contributes to anticipation to a more palatable diet has yet to be investigated.

In order to determine whether the effects of manipulating ghrelin receptor signaling in the DMH were site specific, in the third experiment we administered ghrelin or DLYS-GHRP6 (in place of JMV2959) into the PMV. Both drugs have been extensively used as selective GHSR antagonists, but are structurally distinct: JMV2959 is a 1,2,4-triazole derived small molecule-type competitive antagonist (Moulin et al., 2012), while DLYS-GHRP6 is a peptidergic antagonist (Bowers et al., 1984).

Consistent with our hypothesis, chronic infusion of intra-PMV ghrelin or DLYS-GHRP6 did not affect body weight, fat mass, food intake, or energy expenditure. We did find, however, that in the PMV glucose clearance is slowed in ghrelin treated animals relative to saline but not DLYS-GHRP6 treated animals, similar to the ability of ghrelin infusions into the DMH to slow glucose. This is consistent with the finding that ablation of ghrelin increases glucose-induced insulin secretion, and improves glucose clearance (Sun, Asnicar, and Smith, 2007), and suggests that ghrelin can act in multiple hypothalamic nuclei to induce effects on peripheral insulin and therefore serum glucose. Effects of ghrelin infusions at this site on glucose clearance are consistent with earlier studies show that PMV lesions produce hypoglycemia (Shiriashi and Mager, 1980), and that the PMV projects to areas important for the control of glucose homeostasis, including the PAG and POA (Cabalcante et al., 2006). Thus, stimulation of the ghrelin receptor may be activating neurons in the PMV, that project to areas such as the PAG and POA. However, the mechanisms by which the PMV affects glucose homeostasis are currently not known and require further investigation. Contrary to our hypothesis, both ghrelin and DLYS-GHRP6 infusions into the PMV increased RER values relative to saline treated animals,

indicating that these treatments both increased carbohydrate utilization. This may reflect the fact that DLYS-GHRP6, like JMV2959, has weak agonist properties at the GHSR and can induce intracellular calcium release (Erriquez et al., 2008), that it is a downstream mechanism of calcium signalling that affects fuel utilization. In humans, peripheral DLYS-GHRP6 administration does not mitigate ghrelin-induced GH secretion (Benso et al., 2006). Thus, it is unclear how DLYS-GHRP6 administration is affecting GHSR-mediated processes in our study. Taken together, our data suggest that GHSR signaling in the PMV is important for mediating glucose homeostasis as well as fuel utilization patterns. However, the mechanisms by which this occurs, as well as whether these effects are altered by alterations in energy balance state is not yet known.

Finally, we cannot rule out that the difference in results between JMV2959 treated animals in the DMH and DLYS-GHRP6 treated animals in the PMV is not due to differences in the antagonists used. *In vitro* assays have identified that both antagonists can act as weak agonists, since treatment of human embryonic kidney cells and dorsal root ganglion cells with JMV2959 and DLYS-GHRP6, respectively, induces calcium increases (M'Kadmi et al., 2015; Erriquez et al., 2008). Further, behavioural experiments comparing these two antagonists on ethanol intake in mice indicate that while both antagonists were able to reduce ethanol intake, JMV2959 has a long-lasting effect, whereas DLYS-GHRP6 only reduces intake on the first day, suggesting a quick tolerance (Gomez and Ryabinin, 2014). However, this tolerance is unlikely to account for our lack of effects of DLYS-GHRP6 on food intake or body weight, since not only were there no acute effects of the antagonist on these measures, but respiratory exchange ratio is elevated in these animals relative to saline treated animals, a measure that was taken 5 days after the onset of drug infusion.

Here, we show evidence that chronic infusions of ghrelin into the DMH promotes weight gain and adiposity. 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 ICV ghrelin (Freeman, do Carmo, Adi, and da Silva, 2013; Kamegai et al., 2001), which is known to promote food intake and body weight in rodents. However, here we show that within the DMH, ghrelin acts to promote weight gain and adiposity by limiting energy expenditure, independent of food intake, fuel utilization patterns, and locomotor activity. It is also understood that ghrelin can act on its central receptors to increase peripheral glucose levels (Dimarchi et al., 2014), and in accordance with this, intra-DMH ghrelin attenuates glucose clearance in the glucose tolerance test, a result shared with the effects of intra-PMV ghrelin. In addition, blocking endogenous ghrelin binding in the DMH reduces total caloric intake and promotes carbohydrate utilization and energy expenditure, without affecting body weight. Future studies should investigate the phenotype of the GHSR-positive cells within the DMH and PMV to obtain a better idea of the potential pathways by which ghrelin signaling affects energy balance in these regions. Additionally, electrophysiology would be a useful technique for determining what happens to the firing of the neurons in these regions when agonists or antagonists are present. Lastly, changes in the expression of enzymes mediation non-shivering thermogenesis in BAT of these animals would provide evidence of a potential pathway for how ghrelin and JMV2959 reduce energy expenditure in the DMH.

Appendix

Table 2. Summary of study 2 high-fat diet and chow intake change in each phase. No difference between the groups in these measures.

	Time	Group
Phase 1 HFD	F(4,88)=3.857, p<0.01, η^2 =0.149	F(2,22)=0.104, p>0.05, η^2 =0.009
Phase 2 HFD	F(5.436,119.589)=13.871, p<0.001, η^2 =0.387	F(2,22)=0.093, p>0.05, η^2 =0.008
Phase 3 HFD	F(1.445,58.873)=1.427, p>0.05, η^2 =0.061	F(2,22)=0.028, p>0.05, η^2 =0.003
Phase 2 Chow	F(5.364,118.01)=1.223, p>0.05, η^2 =0.053	F(2,22)=0.043, p>0.05, η^2 =0.004
Phase 3 Chow	F(1.45,31.91)=34.451, p<0.001, η^2 =0.61	F(2,22)=0.113, p>0.05, η^2 =0.010

Table 3. Summary of study 2 metabolic chambers data. No difference between the groups in VCO₂, VO₂, or locomotor activity.

	VCO ₂	VO ₂	Activity
Time Light Phase	F(12.109,159.119)=10.157, p<0.0001, η^2 =0.316	F(11.742,258.322)=4.663, p<0.001, η^2 =0.175	F(15.919,350.219)=11.394, p<0.001, η^2 =0.341
Time Dark Phase	F(11.696,257.313)=5.659, p<0.001, η^2 =0.205	F(12.529,275.640)=4.855, p<0.001, η^2 =0.181	F(14.366,316.041)=11.147, p<0.001, η^2 =0.34
Group Light Phase	F(2,22)=1.344, p>0.05, η^2 =0.109	F(2,22)=0.506, p>0.05, η^2 =0.044	F(2,22)=0.373, p>0.05, η^2 =0.033
Group Dark Phase	F(2,22)=0.026, p>0.05, η^2 =0.002	F(2,22)=1.384, p>0.05, η^2 =0.112	F(2,22)=0.245, p>0.05, η^2 =0.022

Table 4. Summary of study 2 ANCOVAs for RER and energy expenditure.

	ANCOVAs	
	Light Phase	Dark Phase
Energy Expenditure with Lean Mass as a Covariate	F(2,21)=2.587, p>0.05, η^2 =0.198	F(2,21)=4.143, p<0.05, η^2 =0.283
Post-Hoc		Ghrelin vs saline, p<0.05, JMV vs saline, p<0.05
Energy Expenditure with Total Caloric Intake as a Covariate	F(2,21)=7.009, p<0.01, η^2 =0.4	F(2,21)=3.403, p<0.05, η^2 =0.245
Post-Hoc	Ghrelin vs Saline, p<0.01, JMV vs saline, p<0.05	Ghrelin vs saline, p<0.05, JMV vs saline, p<0.05
RER with Chow as a Covariate	F(2,21)=4.142, p<0.05, η^2 =0.283	F(2,21)=1.130, p>0.05, η^2 =0.097
Post-Hoc	Ghrelin vs Saline, p<0.01, JMV vs saline, p<0.05	

Table 5. Summary of study 3 metabolic chambers data. No difference between the groups in VCO₂, VO₂, energy expenditure, or locomotor activity.

	VCO ₂	VO ₂	Activity	Energy Expenditure
Time Light Phase	F(14.14,240.35)=2.865, p<0.001, η^2 =0.144	F(15.7,267.925)=2.02, p<0.05, η^2 =0.106	F(14.181,241.073)=9.349, p<0.001, η^2 =0.355	F(15.412,262.01)=1.707, p<0.05, η^2 =0.091
Time Dark Phase	F(15.203,258.451)=4.132, p<0.001, η^2 =0.196	F(14.904,253.373)=4.656, p<0.01, η^2 =0.215	F(14.991,254.845)=7.062, p<0.001, η^2 =0.293	F(15.73,267.458)=5.038, p<0.001, η^2 =0.229
Group Light Phase	F(2,17)=1.419, p>0.05, η^2 =0.143	F(2,17)=0.935, p>0.05, η^2 =0.008	F(2,17)=2.042, p>0.05, η^2 =0.194	F(2,17)=0.158, p>0.05, η^2 =0.018
Group Dark Phase	F(2,17)=1.772, p>0.05, η^2 =0.173	F(2,17)=0.169, p>0.05, η^2 =0.019	F(2,17)=0.275, p>0.05, η^2 =0.031	F(2,17)=0.017, p>0.05, η^2 =0.002

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