Ghrelin signaling within the paraventricular nucleus of the hypothalamus influences sympathetic activity but not brown adipose tissue activity during the stress response

by

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Abstract

In mammals, ghrelin is secreted during chronic stress to promote the utilization of carbohydrates while decreasing the use of fat as a source for energy. Data suggest that ghrelin secretion during stress may also regulate energy expenditure in the form of heat derived from the activation of brown adipose tissue (BAT). Activation of BAT involves the sympathetic stimulation of non-shivering thermogenesis by the action of uncoupling protein-1 (UCP-1). Furthermore, the paraventricular nucleus of the hypothalamus (PVN) plays a role in energy expenditure by reducing sympathetic outflow on to BAT, thereby decreasing UCP-1 expression, and ultimately decreasing heat production. To do this, mice were implanted with cannulae attached to osmotic minipumps delivering a ghrelin receptor antagonist [D-Lys-3]-GHRP6 (20nmol/day/mouse) or vehicle by the PVN. Half of the mice from each group were subjected to chronic social defeat stress for 19-21 days. Results indicated that stressed animals decreased UCP-1 mRNA expression within BAT, although there were no drug effects. Stressed animals given the antagonist showed increased plasma epinephrine (EP) and norepinephrine (NE) compared to mice in the other groups, but appeared to show less utilization of these in BAT. We therefore suggest that stress alters sympathetic tone to modulate the expression of UCP-1 in BAT and that these effects are not mediated by ghrelin acting on receptors in the PVN.

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List of Abbreviations

α-MSH: α-Melanocyte-stimulating hormone; **β3-ADR:** Beta-3 adrenergic receptor; **AC:** Adenylate cyclase; **ACTH:** Adrenocorticotropic hormone; **ADH:** Antidiuretic hormone; **AgRP:** Agouti related protein; **AMPK:** Adenosine 5-monophosphate-activate protein kinase; **ANOVA-** Analysis of variance; **ARC:** Arcuate nucleus; **ATP:** Adenosine triphosphate; **BAT:** Brown adipose tissue; **CAMP:** Cyclic adenosine monophosphate; **CART**: Cocaine and amphetamine regulated transcript; **CNS:** Central nervous system; **CRH:** Corticotropin-releasing hormone; **DAG:** Diacyl-glycerol; **DMH:** Dorsomedial hypothalamus; **EP:** Epinephrine; **FFA:** Free fatty acids; **GABA:** Gamma-aminobutyric acid; **GC:** Glucocorticoids; **GDP:** Guanosine diphosphate; **GHSR-1a:** Growth hormone secretagogue receptor-1a; **GOAT:** Ghrelin o-acyl-transferase; **GTP:** Guanosine triphosphate; **HPA:** Hypothalamic pituitary adrenal; **HPLC:** High liquid chromatography; **HSL:** Hormone sensitive lipase; **ICV:** Intracerebroventricular; **IML:** Intermediolateral cell column; **IP**: Inositol phosphate; **IP3:** Inositol 1,4,5-tris-phosphate; **KO:** Knock out; **LSD:** Least significant difference; **MCFA:** Medium chain fatty acids; **MHPG**: 3-Methoxy-4-hydroxyphenylglycol; **NE:** Norepinephrine; **NPY:** Neuropeptide Y; **PGC-1α:** Peroxisome proliferator-activated receptor gamma co-activator 1-alpha ; **PIP2:** Phosphatidylinositol 4,5-bisphosphate; **PKA:** Protein kinase A; **PKC:** Protein kinase K; **PLC:** Phospholipase C; **PNS:** Peripheral nervous system; **POMC:** Pro-opiomelanocortin; **PPAR- γ:** Peroxisome proliferator-activated receptor gamma; **PVN:** Paraventricular nucleus of the hypothalamus; **RT-q PCR:** Real time quantitative polymerase chain reaction; **RVLM:** Rostral ventral lateral medulla; **SEM:** Standard error; **SNS:** Sympathetic nervous system; **TG:** Stored triglycerides; **TRH:** Thyroid releasing hormone; **UCP-1:** Uncoupling protein-1; **VTA:** Ventral tegmental area; **WAT:** White adipose tissue; **WT:** Wild type.

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Introduction

The aim of this present study was to investigate the role of ghrelin in modulating the activity of the sympathetic nervous system (SNS) and brown adipose tissue (BAT) during the physiological response to stress. We focused on the paraventricular nucleus of the hypothalamus (PVN), as this region plays an important role in energy homeostasis and regulates autonomic and neuroendocrine functions during the stress response. In addition, we investigated the PVN's connection with the SNS and the activity of BAT. BAT contributes to energy homeostasis through the thermogenic protein, uncoupling protein-1 (UCP-1), by increasing metabolic energy expenditure within the mitochondria (Lowell and Spiegelman, 2000). Therefore, we wanted to examine UCP-1's role in energy expenditure during a negative state, such as chronic social stress. Previous research has focused on the heat generating properties of UCP-1 during cold stress (Meyer *et al.,* 2010). Limited research has looked into UCP-1 activity during social stress. Given that ghrelin targets the PVN to modulate sympathetic function and exogenous ghrelin treatment decreases BAT UCP-1 expression (Yasuda *et al.,* 2003), we hypothesized that ghrelin signaling within the PVN would reduce UCP-1 mRNA expression within BAT.

Physiological Responses to Stress

The stress response is characterized by a number of physiological changes that include hypothalamic pituitary adrenal (HPA) axis activation and sympathetic activation. These allostatic physiological mechanisms enable the organism to deal with the energetic threat by utilizing available reserves (McEwen, 2007; Patterson *et al.,* 2013a). The HPA axis is activated when neurosecretory cells within the PVN secrete corticotropin-releasing hormone (CRH) into the hypophyseal portal network that travels from the median eminence to the anterior pituitary gland where CRH stimulates the secretion of adrenocorticotropic hormone (ACTH) into the general circulation. Once in circulation, ACTH acts directly on the adrenal cortex to stimulate the release

of glucocorticoids (GCs). The main GCs are corticosterone in rodents or cortisol in humans. Glucocorticoids then have a number of effects that are important to meet the energetic challenges of a stressor. For example, GCs increase glucose availability by stimulating gluconeogenesis (Schacke *et al.,* 2002). Excess GCs also stimulate the release of lipids from adipose tissue and promote lipid catabolism in peripheral cells (Schacke *et al.,* 2002). These effects are advantageous in the short term but in the long term they can lead to undesirable effects associated with metabolic disturbances such as obesity or hyperglycemia.

Ghrelin

In addition to the stimulation of the HPA axis, the stress response includes the release of hormones that regulate energy balance. One of the hormones that is released in response to stress is ghrelin. Ghrelin is a 28 amino acid hormonal peptide that plays an important role in energy metabolism by regulating food intake, body weight, and glucose homeostasis (Kojima *et al.,* 1999). These biological effects are dependent on a number of post-translational modifications to the structure of the translated ghrelin protein. The immature ghrelin peptide is first cleaved by protein convertase-1 producing desacyl ghrelin and obestatin, an anorexigenic peptide. Desacyl ghrelin is then acylated by Ghrelin-O-Acyltransferase (GOAT), a modification that is required for the biological properties of ghrelin (Sakata *et al.,* 2009). Interestingly, GOAT levels rise after ingesting foods containing high amounts of medium chain fatty acids (MCFA) suggesting that high fat diets may increase the amount of active ghrelin being secreted (Sakata *et al.,* 2009). Once acylated, ghrelin is secreted from the gastric lamina propria of the stomach by X/A-like cells within the oxyntic glands of the gastric fundus mucosa. Acylated ghrelin travels through the bloodstream and binds to target tissues expressing its only known receptor: the growth hormone secretagogue receptor-1a (GHSR-1a).

GHSR-1a is a G-protein-coupled 7-transmembrane receptor and when ghrelin binds to this receptor, several signal transduction pathways occur, including the inositol phosphate (IP) signaling pathway through phospholipase C (PLC) activation (Adams *et al.,* 1995; Lei *et al.,* 1995; Chen *et al.,* 1996). GHSR-1a is composed of three different subunits, α, β and γ, where α-subunit has a guanine nucleotide binding pocket. When ghrelin binds to this pocket, the receptor changes the shape of the α-subunit allowing guanosine diphosphate (GDP) to readily dissociate and the nucleotide pocket is then occupied by guanosine triphosphate (GTP). Since GHSR-1a interacts with the αq-subunit (Gαq), this subunit activates PLC, which cleaves phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates protein kinase C (PKC), which in turn activates Ca^{2+} channels and inhibits K^{+} channels.

Within the brain, the hypothalamus has the highest level of expression of GHSR-1a. A number of hypothalamic nuclei including the arcuate nucleus (ARC), PVN, dorsomedial hypothalamus, ventromedial hypothalamus, and lateral hypothalamus show an increase in the number of c-Fos-positive cells following intracerebroventricular (ICV) infusions of ghrelin (Olszewski *et al.,* 2003; Kamegai *et al.,* 2001). These effects were also associated with increased food intake, bodyweight regulation, and glucose homeostasis (Nakazato *et al.,* 2001). Furthermore, studies with GHSR-1a KO have shown that a lack of ghrelin signaling fails to acutely stimulate food intake (Zigman *et al.,* 2005). Even when fed a high fat diet, GHSR-1a KO mice ate less food, stored less of their consumed calories in adipose tissue, preferentially utilized fat as an energy substrate, and accumulated less body weight and adiposity than control mice (Zigman *et al.,* 2005).

In addition to the hypothalamus, GHSR-1a is expressed in a number of brain regions, such as limbic structures that are important for motivational and emotional aspects of feeding behavior. One of these is the ventral tegmental area (VTA), a region containing neurons that produce dopamine and are associated with reward seeking behaviors. Abizaid and colleagues (2006) showed that direct VTA administration of ghrelin increased food intake and also increased dopamine neuronal activity (Abizaid *et al.,* 2006). This demonstrates that ghrelin targets the mesolimbic dopaminergic system to increase food consumption (Abizaid *et al.,* 2006). Furthermore, ghrelin infused in the VTA has also been associated with increases in the intake and motivation to obtain palatable foods (King *et al.,* 2011; Perello *et al.,* 2010). Given that rats exposed to comfort foods show significantly lower CRH mRNA expression in the hypothalamus, these data support the idea that ghrelin stimulates the intake of foods that are palatable and reinforcing, which thereby reduce the activity of the HPA axis (reviewed by Dallman *et al.,* 2003).

Ghrelin and stress

Evidence from our lab and others have established that ghrelin is also involved with the stress response (Asakawa *et al.,* 2001; Lutter *et al.,* 2008; Patterson *et al.,* 2010). Plasma ghrelin levels rise in response to acute and chronic stressors (Asakawa *et al.,* 2001). Interestingly, animals exposed to repeated social stressors often showed increased caloric intake, body weight, and adiposity (Lutter *et al.,* 2008; Bartolomucci *et al.,* 2009, Patterson *et al.,* 2013b). Patterson and colleagues (2013) showed that GHSR KO mice or C57BL/J6 mice receiving ICV infusions of [D-Lys3]-GHRP6 had similar decreased weight gain and feeding responses under chronic social defeat stress compared to WT or vehicle infused stressed mice (Patterson *et al.,* 2013b). It was therefore concluded that chronic social stress produced a number of metabolic effects and these were mediated by ghrelin acting on GHSR-1a located in the brain (Patterson *et al.,* 2013b).

One thing that remains unclear is the relative contribution of each brain region to the effects of ghrelin under stressful conditions.

The Paraventricular Nucleus

In addition to the ARC and the VTA, ghrelin receptors are found in other brain regions that are important for food intake, energy balance, and stress. One of these regions is the PVN, a hypothalamic nucleus that is important in the regulation of energy homeostasis and where ghrelin signaling within the PVN is part of the stress response. The PVN is a bilateral nuclei that sits on the dorsal portion of the third ventricle and lies posterior to the preoptic area and is anterior to the ARC and ventromedial nucleus of the hypothalamus. The major target neurons within the PVN and their actions are outlined Table 1. The PVN contains two major subdivisions, the magnocellular and parvocellular divisions.

Table 1: Major target neurons within the PVN and their actions.

TARGET NEURONS WITHIN PVN	SUBDIVISION	SYSTEM ACTIVATING	RESPONSE AREAS
PARVOCELLULAR	Neurosecreting	Endocrine System	Corticotropin-releasing hormone
	Autonomic system	Sympathetic Nervous System	Adrenal cortex Brown adipose tissue White adipose tissue
MAGNOCELLULAR	Neurosecreting	Endocrine System	Vasopressin Oxytocin

Magnocellular neurons located within the PVN project directly to the posterior pituitary cells and secrete oxytocin and vasopressin. Oxytocin is known for smooth muscle contraction of the uterus promoting labor yet increasing evidence suggests a role during chronic stress (Uvnas-Moberg and Petersson, 2005). Oxytocin is upregulated during acute stress and remains elevated during chronic stress (Zheng *et al.,* 2010). Uvnas-Moberg and Petersson suggested that oxytocin

facilitates an adaptive role during chronic stress by inducing anti-stress effects, such as decreasing blood pressure and increasing pain threshold (Uvnas-Moberg and Petersson, 2005). Vasopressin, also known as antidiuretic hormone (ADH), is released in response to elevated solute concentrations or decreased blood volume (Leaf and Hays, 1962). The primary function of ADH is to decrease water lost in the kidney. During stress; however, ADH levels rise which elevates arterial blood pressure by constricting peripheral blood vessels resulting in an elevated heart rate and an increase in water retention (Rogers and Hermann, 1985).

Parvocellular cells located within the PVN can be further subdivided into sympathetic (autonomic) and neurosecretory divisions. Parvocellular neurosecretory cells secrete CRH into the hypophyseal portal system and activate corticotrophs in the anterior pituitary gland to secrete ACTH, as mentioned previously. During acute stress, CRH positive cells show increased mRNA expression within the PVN, yet during chronic stress there is a significant decrease in CRH expression (Zheng *et al.,* 2010) that is due to glucocorticoids exerting a negative feedback signal (Vandenborne *et al.,* 2005). Interestingly, centrally administrated ghrelin stimulates CRH mRNA expression in the hypothalamus, activating the HPA axis. This subsequently increases ACTH and glucocorticoid plasma levels in rodents (Wren *et al.,* 2002; Stevanovic *et al.,* 2007). The effects of ghrelin on CRH secretion appear to be indirect as CRH neurons do not appear to express ghrelin receptors (Cabral *et al.,* 2012).

Parvocellular autonomic neurons in the PVN project to the sympathetic preganglionic neurons in the intermediolateral (IML) cell column of the spinal cord and/or the rostral ventrolateral medulla (RVLM). The IML of the thoracolumbar spinal cord contains the sympathetic preganglionic motor neurons and this innervation lies primarily in the lower thoracic/upper lumbar region where sympathetic preganglionic neurons innervate the periphery.

Studies show that neurons in the PVN projects to the spinal cord and synapse directly on to sympathetic preganglionic motor neurons (Csaki *et al.,* 2000). The RVLM remains the primary regulator of the SNS, sending excitatory fibers to sympathetic preganglionic neurons in the spinal cord that give rise to the sympathetic nerves. The RVLM contains neurons that project to and densely innervate the sympathetic preganglionic motor neurons. This region is essential for the reflex sympathetic nerve responses that is elicited by many cardiovascular stimuli and is essential for the generation of sympathetic tone (Csaki *et al.,* 2000). These sympathetic nerves regulate heart rate, blood pressure, BAT, and the activity on the adrenal gland. Activation of these circuits results in an elevated release of epinephrine (EP) and norepinephrine (NE) into the circulation (Bhatnagara and Vininga, 2003). Neurons in the PVN inhibit BAT likely via the activation of GABAergic cells in the premotor neurons of the raphe pallidus, which project to BAT (Madden and Morrison, 2009). However, the role of the PVN in regulating thermogenesis and energy expenditure in BAT remains to be fully determined.

Afferents to the PVN

The PVN receives innervation from catecholaminergic (adrenergic or noradrenergic) axons originating from the caudal medulla and locus coeruleus. In addition, the PVN receives peptidergic ascending fibers containing neurotensin, neuropeptide Y, bombesin, inhibin beta, enkephalin, and somatostatin. These projections originate from brain stem regions, like the nucleus of the solitary tract and the parabrachial nuclei (Affleck *et al.,* 2012). The PVN also receives ascending serotonergic fibers from the dorsal and midbrain raphe nuclei. Lastly, the PVN receives dense projections from the ARC and these are critical in the regulation of food intake and energy balance (Aponte *et al.,* 2011). The projections from the ARC stem from neuropeptide secreting cells that have orexigenic (NPY, and AgRP) or anorexigenic (α-MSH)

effects as well as peripheral hormones, such as ghrelin and leptin, that can influence the release on these neuropeptides.

PVN and food intake

The PVN, like the ARC, contains peptidergic cell groups that have been associated with either increased or decreased food intake. In addition, the PVN contains receptors for orexigenic and anorexigenic peptides that are secreted from the ARC. For example, neuropeptide Y (NPY) and agouti-related protein (AgRP) are released and stimulate receptors within the PVN to increase appetite and decrease energy expenditure. The orexigenic effect of these peptides was dramatically demonstrated in mice that have their NPY/AgRP neurons selectively destroyed. These mice decreased their food intake to the point of starvation (Luquet *et al.,* 2005). GHSR is primarily expressed in AgRP /NPY neurons and ghrelin increases the transcription and release of both of these peptides (Cowley *et al.,* 2003). Ghrelin activates adenosine monophosphate (AMPK) activated protein kinase in the hypothalamus and specifically activates AMPK in NPY neurons within the ARC (Kamegai *et al.,* 2001). AMPK acts as a cellular energy sensor that responds to low ATP levels, such as chronic stress or food deprivation (Mihaylova and Shaw, 2011). AMPK activation positively regulates signaling pathways that replenish cellular ATP supplies, including fatty acid oxidation. AMPK negatively regulates ATP-consuming biosynthetic processes including gluconeogenesis, lipid and protein synthesis (Mihaylova and Shaw, 2011).

Within the PVN, however, there are cells that produce and release orexigenic peptides other than NPY and AgRP. One of these is galanin, a peptide that has a potent stimulatory effect on eating behavior following acute ICV (Kyrkouli *et al.,* 1986) and intra-PVN infusion (Kyrkouli *et al.,* 1990). This shows that galanin affects metabolism, causing a reduction in

energy expenditure (Menendez *et al.,* 1992). Studies have shown that galanin injections within the PVN have found a preferential increase in the consumption of the fat diet (Tempel *et al.,* 1990). This suggests that endogenous galanin contributes to the natural appetite for fat (Tempel *et al.,* 1990).

In contrast, the PVN also mediates the anorectic effects of pro-opiomelanocortin (POMC) and Cocaine and Amphetamine Regulated Transcript (CART) (Holst and Schwartz, 2004) producing cells in the ARC. Mutations to the POMC gene result in morbid obesity and decreased energy expenditure (Gropp *et al.,* 2005). In addition, ghrelin indirectly decreases POMC neuron activity via local gamma-aminobutyric acid (GABA) release. In this case, ghrelin stimulates local release of GABA from AgRP/NPY neurons and this binds to GABA receptors located on POMC neurons (Riediger *et al.,* 2003). Finally, CRH, oxytocin, and TRH secreting neurons in the PVN are known to produce anorectic effects (Sarkar *et al.,* 2002). Given that the PVN can have both orexigenic and anorectic effects, it has been suggested that that PVN is a region that influences feeding, emotional, and metabolic responses.

PVN sympathetic innervation to WAT

The PVN contributes to energy balance regulation in part by modulating lipolytic/lipogenic mechanisms in response to the changing metabolic state. The PVN indirectly modulates white adipose tissue (WAT) activation, as demonstrated by viral transneuronal retrograde tract tracers. For instance, unilateral viral tracer injections into inguinal WAT of Siberian hamsters resulted in predominantly unilateral labeling the PVN ipsilateral to the injected inguinal WAT pad. During fasting, triglycerol stores are mobilized while fatty acids and glycerol are released into the bloodstream, all of this through connections between the PVN and adipose tissue. Electrical stimulation of the postganglionic SNS fibers markedly increases FFA

concentration (Correll, 1963). This effect is blocked with a β-adrenergic receptor antagonist prior to electrical stimulation and stimulated by adrenal medullary catecholamines (Weiss and Maickel, 1965). Lastly, PVN lesions trigger anabolic responses that result in increased body and WAT mass (Bamshad *et al.,* 1998; Song *et al.,* 2005).

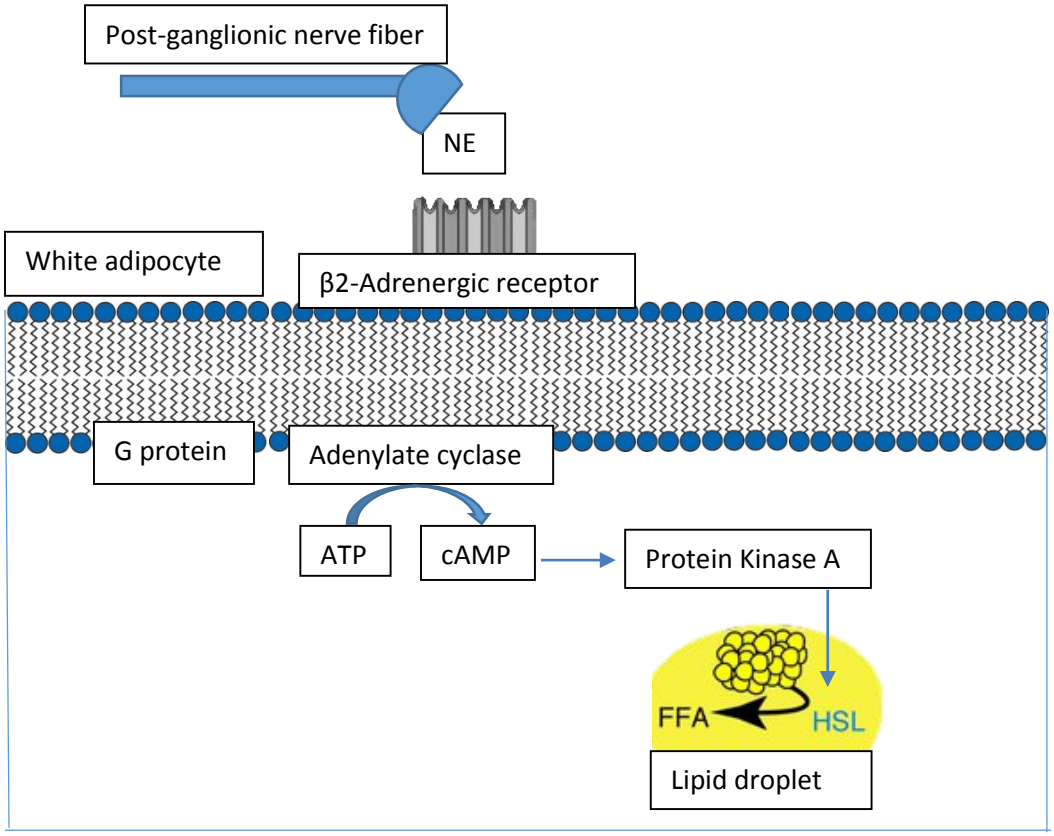

Figure 1: Diagram outlining sympathetic post-ganglionic nerve effects on white adipose tissue lipolysis and hormone sensitive lipase

As seen in Figure 1, NE is released from postganglionic nerve endings and this neurotransmitter binds to β2 adrenergic receptors on the WAT. This triggers intracellular G protein signalling pathways that activate adenylate cyclase (AC) and subsequently protein kinase A (PKA). PKA activity phosphorylates hormone sensitive lipase (HSL), which is the rate limiting enzyme involved in lipid mobilization and lipolysis. HSL hydrolyzes stored triglycerides

into free fatty acids and glycerol. Free fatty acids are transported to the plasma membrane bound to adipocyte fatty acid-binding protein and then are transported across the plasma membrane into the circulation. Lipolytic processes within WAT have been extensively studied and is similar to BAT lipolytic processes.

Brown adipose tissue BAT as a source of energy

The existence of BAT was first reported in 1551 (Gessner, 1551) yet the realization that BAT is found in all mammals only occurred within the last century (Hatai, 1972). Its thermogenic properties were first shown in hibernating animals (Smith, 1961) although there were some early reports of the existence of brown fat in humans (Hatain, 1902). Only recently has it been demonstrated that BAT is important for energy metabolism in adult humans (Lidell *et al.,* 2013). In humans, BAT is primarily found in newborns and infants, due to their inability to adequately conduct shivering thermogenesis. After infancy, BAT mass diminishes yet BAT still persists into adulthood (Heaton, 1972). In addition, estimates suggest that as little as 40 g of maximally stimulated BAT could account for 20% of daily expenditure in an adult human (Rothwell and Stock, 1983). The activation of BAT and its ensuing generation of heat are mediated by the activity of the sympathetic nervous system (Smith, 1961). Given that this system is stimulated during the stress response, it is possible that stress could impact energy expenditure through the activation of BAT by EP of NE.

Molecular mechanism of UCP-1

Post-ganglionic nerves secrete NE that binds to β3-adrenergic receptors located on BAT. This interaction activates adenylate cyclase (labeled as AC in Figure 2) increasing cAMP (Cyclic adenosine monophosphate) and PKA activity. PKA triggers HSL, showing a similar mechanism from WAT activation. HSL stimulates the hydrolyzation of stored triglycerides (TG) that liberate fatty acids (FFA). These FFA become oxidised in the mitochondria, resulting in the rapid

induction of uncoupling protein-1. This protein uncouples oxidative phosphorylation and the energy from the proton motive force is dissipated as heat rather than producing ATP (Yasuda *et al.,* 2003). UCP-1 expression and its activity is a metabolically expensive pathway, thereby promoting energy expenditure (Seale *et al.,* 2007). Furthermore, specific ablation of UCP-1 leads to reduced whole-body energy expenditure (Hermann *et al.,* 1996). Thus, UCP-1 allows for a transmembrane flow of ions. The mechanism could either be described as a carrier or a channel. A channel would allow bulk flow whereas the carrier would move one molecule of the transported species per turnover. Both models have been suggested, due to the similarity of UCP-1 to the ATP/ADP carrier, a carrier model has often been promoted but a channel has also been suggested due to an observed linear effect of membrane potential on transport rate and the high transport number at high potential (as reviewed by Klingenberg, 2008).

There is some question whether these fatty acids are the only main substrates inducing UCP-1 activation. HSL-deficient mice fail to show BAT lipolysis (Wang *et al.,* 2001). These animals show greater lipid vacuoles in white and brown adipocytes than in the wild type, further indicating that lipolysis is diminished (Wang *et al.,* 2001). Wang and colleagues suggested that HSL is both responsible and obligatory for norepinephrine-mediated lipolysis in brown adipocytes. They further noted that there was a normal rate of lipolysis in unstimulated HSL−/− adipocytes and they suggested that HSL-independent lipolytic pathway may exist in BAT (Wang *et al.,* 2001). It is generally accepted that FFA are the main activators of UCP-1 activity.

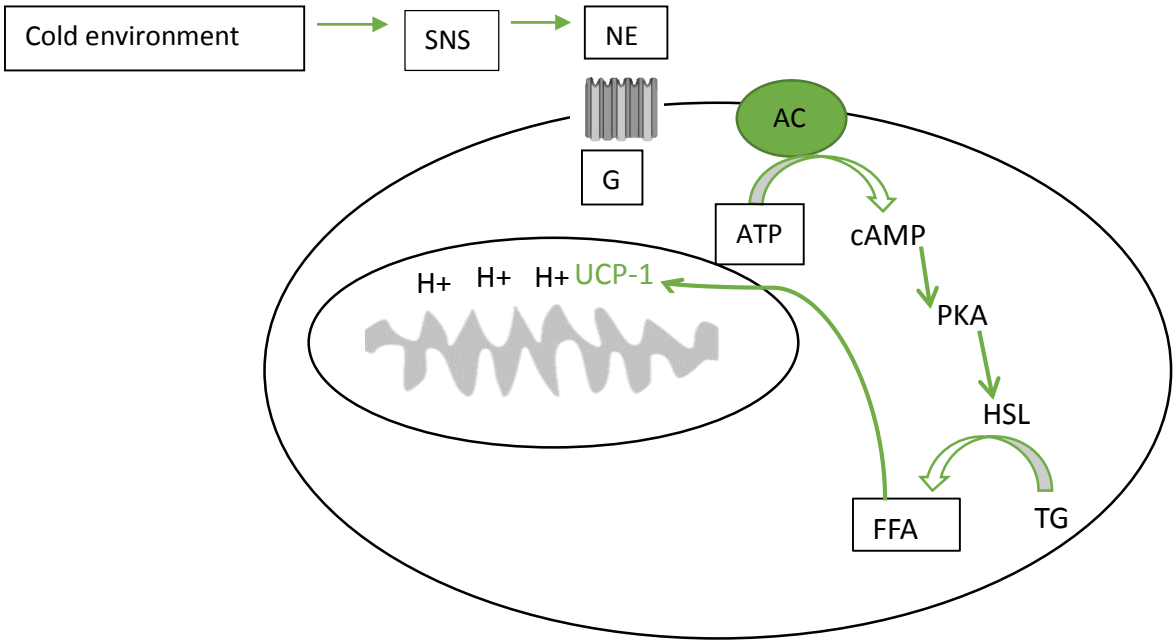

Figure 2: UCP-1 and brown adipose tissue thermogenesis signaling.

UCP-1's thermogenic capacity

The thermogenic capacity of BAT is determined by recruitment (determined by total number of brown adipocytes in the tissue by proliferation and apoptosis) plus activity (degree of differentiation of the tissue, including the mitochondrial density and amount of UCP-1). The degree of activity is determined by the acute rate of sympathetic stimulation (NE release) and the recruitment state is mainly determined by the chronic level of sympathetic stimulation (Cannon and Nedergaard, 2004).

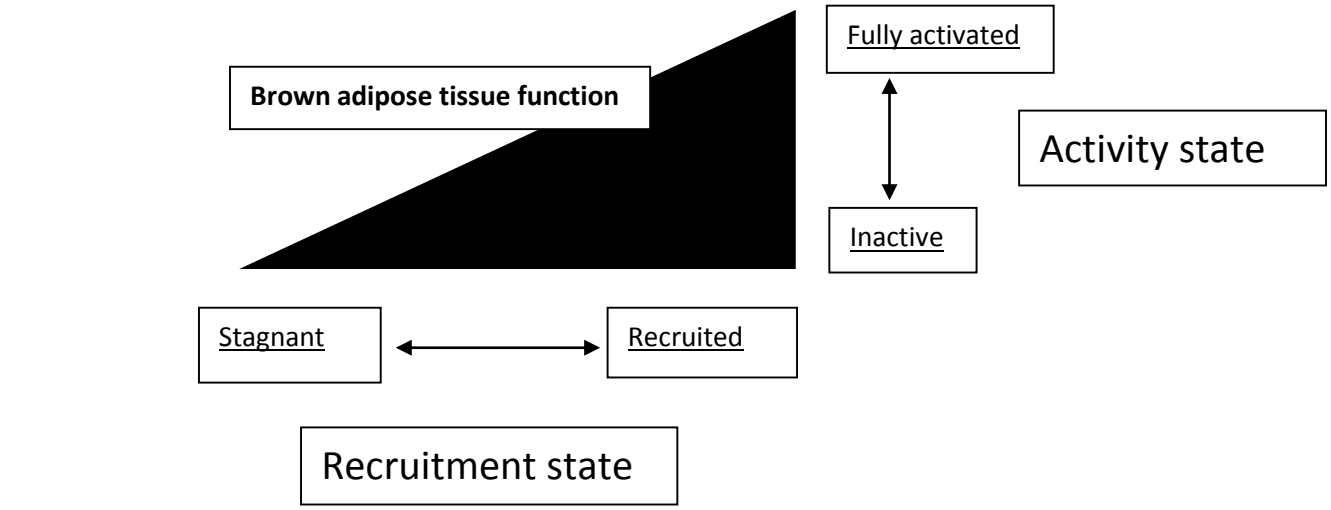

Figure 3: Activity and recruitment state of brown adipose tissue. Adapted from (Cannon and Nedergaard, 2004).

Recruitment of BAT

Acute cold exposure leads to a rapid delipidation of BAT as mobilization and oxidation of lipid stores reduce the capacity of brown adipocytes to take up or synthesize lipids (Holm *et al.,* 1987). The first physiological adaptation to cold exposure in BAT is an increase in lipid uptake. On a transcriptional level, expression of HSL, which is essential for lipid uptake into BAT, is induced as early as one hour after cold exposure (Holm *et al.,* 1987). Conversely, UCP-1 KO mice exhibit greater BAT lipid content than wild-type mice at room temperature (Enerback *et al.,* 1997).

Recruitment of BAT involves the total number of brown adipocytes, including the rate of proliferation and apoptosis. Numerous studies have shown chronic NE stimulation of brown preadipocytes and mature adipocytes will lead to a recruitment state (Bouillard *et al.,* 1984). The

adrenergic effects indicate that the recruitment process is promoted under conditions of a constant demand for thermogenesis by increasing energy expenditure during times of excess energy.

UCP-1 activity: Mitochondrial biogenesis

Mitochondrial biogenesis within BAT is strongly activated by cold stress and results in a three-fold increase in the amount of mitochondrial protein per brown adipocyte (Rafael *et al.,* 1985; Klingenspor *et al.,* 1996b). The transcriptional coactivator, peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α), is regarded as the master regulator of mitochondriogenesis, by interacting with the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) that then activate mitochondrial DNA replication and transcription. Uldry and colleagues used PGC-1α knockdown mice and found the loss of PGC-1α severely reduced the induction of thermogenic genes (Uldry *et al.,* 2006). The connection between ghrelin and PGC-1α has not been fully elucidated. Studies have shown chronic peripheral ghrelin suppresses UCP-1 expression (Tsubone *et al.,* 2005) yet it is not known if PGC-1α mediates this suppression. In addition, Lehr and colleagues (2006) showed that the control of UCP-1 is dissociated from that of PGC-1α function (Lehr *et al.,* 2006). Studies do indicate PGC-1α null mice showed reduced mitochondrial function and had impaired physiological processes that rely on mitochondrial metabolism.

UCP-1 activity and high fat food

Another factor that alters UCP-1 expression in BAT is food consumption. It is well know that there is a postprandial increase in body temperature, often referred to as obligatory diet-induced thermogenesis (Rothwell and Stock, 1979). This diet-induced thermogenesis can be defined as the increase in energy expenditure above basal metabolic food divided by energy content of food ingested. Yet during an excessive amount of food intake, there is an exaggerated

induction of thermogenic pathways that results in elevated heat production. To combat obesity, essentially two alternatives exist: Decrease energy intake or increase energy expenditure. Chronic intake of certain diets in itself augments the capacity for increased energy expenditure from the thermogenic pathway within BAT. Interestingly, UCP-1 ablation leads to increased susceptibility to obesity (Jung *et al.,* 1979; Kontani *et al.,* 2005; Feldmann *et al.,* 2009).

Rationale for thesis

Given that chronic psychological stress produces a significant energetic challenge, it is reasonable to propose that psychological stress modulates UCP-1 expression in BAT. Furthermore, since peripheral ghrelin modulates UCP-1 expression (Yasuda *et al.,* 2003) and ghrelin is released in response to stress, one could argue that if stress modulates UCP-1 expression it does so through the release of ghrelin (Mano-Otagiri *et al.,* 2009). Finally, given that ghrelin seems to affect energy expenditure in response to stress by central pathways (Patterson *et al.,* 2013b; Tschop *et al.,* 2000), one would expect that ghrelin acts at central sites to modulate UCP-1 expression in BAT. Of these sites, we have chosen to investigate the PVN, as this region influences sympathetic activation of BAT (Mano-Otagiri *et al.,* 2009).

We therefore propose that stress induced ghrelin secretion decreases BAT activity, as reflected in a decrease in UCP-1 expression and suppressed noradrenergic tone in BAT. This effect is mediated through the action of ghrelin signaling within the PVN. To test this hypothesis, we conducted an experiment where a ghrelin receptor antagonist was infused by the PVN in chronically stressed and non-stressed mice. We compared their NE turnover rates and UCP-1 mRNA expression in BAT with vehicle stress or non-stressed controls. We predicted that stress would decrease the expression of UCP-1 in BAT and furthermore, that ghrelin receptor antagonist infusion into the PVN would block this effect.

Materials and Methods

Animals

Male C57/BL6 mice (N = 48) weighing 20-22 grams were obtained from Charles River farms, St. Constant, Quebec as experimental subjects. These mice served as the experimental animals. Additional male CD-1 retired breeder mice weighing 40-50 grams were used as experimental subjects for stressors in the chronic social defeat stress paradigm. All mice were housed under standard laboratory conditions with *ad libitum* access to mouse chow and tap water in addition to daily 4-hour high fat diet containing 60% caloric content from fat (TD 06414, Harlan). The calculated metabolized energy of the high fat diet was 5.1 kcal/g with 60% calories from fat while the chow showed 3.3 kcal/g with 70% calories from carbohydrates. The procedures documented were approved by the Carleton University Animal Care Committee and the guidelines of the Canadian Council on Animal Care were followed.

Stereotaxic surgery of chronic paraventricular nucleus of the hypothalamus delivery of ghrelin receptor antagonist

The body weight, chow, and high fat intake were monitored daily for five weeks and these data served as a baseline for the rest of the study. At the end of the baseline, mice were anesthetized using isofluorane mixed with oxygen using an anesthesia circuit mask (Kopf Instruments, Tujunga, CA). While anesthetized, the mouse's head was shaved and secured onto a mouse stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The scalp was cleaned with surgiprep and privodine to provide an aseptic canvas. Tear gel was also applied to prevent dehydration of the eyes. A midline incision was made and the skin was retracted for a clear visualization of bregma. A 28 gauge stainless steel unilateral cannula (Alzet Brain Infusion Kit #0004760; flow rate: 0.25µL/hr for 28 days) coupled to an osmotic mini-pump (Alzet Mini-Osmotic Pump Model 2ML4) using a polyethylene catheter and was implanted into the paraventricular nucleus (PVN) of the hypothalamus. Stereotactic coordinates of the cannula,

relative to bregma were, AP -0.94 mm, ML -1.75 mm and DV 4.83 mm. Mini-pumps were filled with 240µL of either sterile saline (0.9% NaCl) or ghrelin receptor antagonist ([D-Lys3]-GHRP-6; Peptides International; 20nmol/day/mouse). The implant was secured with contact and dental cement. When the cement was dry, the portion of the skin closest to the head cap was further cut by about 1 cm, the skin was gently separated from the muscle and the mini-pump was implanted subcutaneously into the interscapular space. The incision was closed using surgical staples. Polysporin and Lidocaine were applied topically to the surgical site to prevent bacterial infection and pain, respectively. Mice were also injected with a low dose of meloxicam (Metacam, 5mg/kg) to provide postoperative analgesia. Animals were allowed to recover for an additional 7-10 days before the start of the chronic social defeat paradigm.

Chronic Social Defeat Stress Paradigm

After recovering from the surgery period, vehicle and ghrelin receptor antagonists infused animals were assigned to a stress or non-stress condition. Mice in the stressed groups were transported to a separate room and subjected to social defeat stress daily for 19-21 days. In this paradigm, each experimental mouse was placed into the cage of a larger, sexually experienced CD-1 mouse. Mice were allowed to interact with the resident aggressive mouse for 15 minutes or until the experimental mice showed a submissive pose (i.e. stand in boxing position, backing out). Great care was taken to avoid physical injury to the experimental animals and none were physically injured during the duration of the study. Once the interaction was terminated, mice were allowed to co-habitat in the same cage but they were separated by an acrylic divider with a wire mesh allowing for olfactory, visual, and auditory contact between the two mice. Each day, the divider was removed and the animals were permitted to interact for 15 minutes or until the experimental animal showed signs of submission. This was repeated daily for 19-21 days. At the end of the stress period, animals were sacrificed the morning after their last period of interaction

with the aggressive CD-1 mouse. Control non-stressed mice were sacrificed at an experimental time similar to that of stressed animals.

Tissue Processing

Animals were sacrificed by rapid decapitation as plasma and tissue samples were collected and frozen at -80°C. Trunk blood was deposited in borosilicate tubes coated with EDTA and chilled on ice before centrifugation. Blood samples were then centrifuged at 3,000 g for 15 minutes at 4°C to separate red blood cells from plasma. Plasma samples were aliquoted and stored at -80°C for future analysis. BAT was collected and stored in Trizol at -80°C for RT-qPCR analysis. Brains were rapidly dissected and placed in tubes with 4% PFA for 72 hours then placed in 30% sucrose solution. The brains were stored at 4°C for approximately 48 hours or until the brains had sunk to the bottom of the vial. The brains were placed to the sectioning block of the cryostat (LEICA CM1900) with HistoPrep. Carcasses were stored in -80°C until they were processed to measure the weight of different fat pads. To do this, frozen carcasses were later thawed, dissected, and weighed measuring retroperitoneal, perigonadal, subcutaneous, and BAT.

High Liquid Chromatography (HPLC) Analyses

HPLC procedure was analyzed for amine and metabolite levels in plasma and within BAT. Levels of NE, EP, and 3-methoxy-4-hydroxyphenylglycol (MHPG) were determined by HPLC using a modification of the method by Seegal et al. (1986). Using a Waters Associates (Milford, MA) M-6000 pump, guard column, radial compression column (5m, C18 reverse phase, 8 mm 3 10 cm), and three cell colorimetric electrochemical detector (ESA model 5100A), 20 ml of the supernatant was passed through the system at a flow rate of 1.5 ml/min (1400–1600 psi). The mobile phase was used for the separation of our sample. Each liter consisted of 1.3 gm of heptane sulfonic acid, 0.1 gm of disodium EDTA, 6.5 ml of triethylamine, and 35 ml of acetonitrile. The mobile phase was then filtered (0.22 mm filter paper) and degassed, then the pH

was adjusted to 2.5 with phosphoric acid. The area and height of the peaks was determined using a Hewlett–Packard (Palo Alto, CA) integrator. The protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, Ontario, Canada) and a spectrophotometer (PC800 colorimeter; Brinkmann Instruments, Westbury, NY). The lower limit of detection for the monoamines and metabolites was 5.0 pg/mg of protein.

Real Time q-PCR

Total RNA from BAT was isolated with TriZol and formed a precipitate with 13µl of linear acrylamide. RNA quality and concentrations were determined by absorbance at 280 nm and 260 nm with a Thermo Scientific Nanodrop 100 spectrophotometer (Thermo Scientific, Rockford, Illinois). To synthesize cDNA, 1 µl oligo (dT) primer (Invitrogen, Carlsbad, California) was added to 9µl of mRNA and heated to 70°C for 5 minutes. To each sample, a master mix composed of 4µl of 5" first-strand buffer (Invitrogen), 2µl of 0.1 M dithiothreito (Invitrogen), 1 µl of RNase inhibitor (Promega Corp, Madison, Wisconsin), 1 µl of 10 mM deoxynucleotide triphosphate (Invitrogen), 1 µL of diethylpyrocarbonate water, and 1 µl of SS2 reverse transcriptase (Invitrogen) were added. Samples were then run on a PTC-200 Thermal Cycler (MJ Research, Watertown Massachusetts) at 42°C for 1.5 hours followed by 90°C for 10 minutes. Samples were stored at -20°C. Then, 5µl of each cDNA sample was added to separate wells in a PCR plate. Two microliters of working primer solution, 3µl of Milli-Q water, and 10 µl of iQ SYBR Green Super Mix with fluorescein (Bio-Rad Laboratories, Inc, Hercules, California) were added to each well. Samples were run in duplicate, with non-template controls. The plate was run on a MyIQ Single Color Real-Time PCR Detection System (Bio-Rad) for 30 sec at 95°C, followed by 45 cycles of the following settings: 10 sec at 95°C for denaturing, 30 sec at 55°C for annealing, and 20 sec at 72°C for extension. The plate was then run for 1 minute

at 95°C and 1 minute at 55°C. RT-qPCR was conducted on all cDNA samples to determine fold changes using the $2^{-\Delta\Delta Ct}$ method. This method used primers detecting housekeeping genes, such as glyceraldehyde 3-phosphate dehydrogenase gene, β-actin, and 36B4. These housekeeping genes were used as a control transcript to ensure there were no differences in cDNA purity between experimental groups. The $2^{-\Delta\Delta Ct}$ method also compares gene expression between a treatment condition (drug or stress) relative to a control (non-stress vehicle).

Primer sequences used for the real-time qPCR:

PGC-1α-F: 5' AGC CGT GAC CAC TGA CAA CGA G-3'

PGC-1α-R: 5' GCT GCA TGG TTC TGA GTG CTA AG-3'

UCP-1-F: 5' GAT CCA AGG TGA AGG CCA GG-3'

UCP-1-R: 5' GTT GAC AAG CTT TCT GTG GTG G-3'

HSL-F: 5'-CCT ACT ACA CAA ATC CC-3'

HSL-R: 5'-CTC AAA GAA GAG CAC TC-3'

β3-ADR-F: 5′-CAG CCA GCC CTG TTG AAG-3′

β3-ADR-R: 5'-CCT TCA TAG CCA TCA AAC CTG-3′

β-ACTIN-F: 5'-TCA TGA AGT GTG ACG TTG ACA TCC G-3'

β-ACTIN-R: 5'-CCT AGA AGC ATT TGC GGT GCA CGA TG-3'

GAPDH-F: 5'-AAG ATG GTG AAG GTC GGT GT-3'

GAPDH-R: 5'-CTT GCC GTG GTA GAG TCA T-3'

36B4-F: 5’-GGC CCG AGA AGA CCT CCT T-3’

36B4-R: 5’-TCA ATG GTG CCT CTG GAG GAT T-3’

All primers were tested for amplification efficiency using the standard curve method, yielding efficiencies over 90%.

Statistics

Mice were assigned to different experimental groups according to weight and average caloric intake before the surgeries were conducted. Average caloric and weight gain data during the surgery recovery period were analyzed using a one-way ANOVA to determine differences between drug and vehicle treated animals. Average caloric intake, chow intake, high fat intake and body weight data during the stress period were analyzed using two-way ANOVA with drug ([D-Lys-3]-GHRP6 vs. 0.9% saline) and treatment (stress vs. non-stress) as between group factors. Fisher's least significant difference post hoc tests were performed if there was a significant interaction effect. Similar analyses were conducted for fat pad weight, mRNA expression, and catecholamine measures. The limit for statistical significance was set at $\alpha = 0.05$.

Results

From the starting 48 animals used in this study, two died during surgery and 10 additional mice had to be removed during the recovery period given that they showed signs that met the endpoints specified in the surgical procedures (i.e. decreased feeding, poor grooming, and weight loss), therefore we deemed these animals not suitable for the study. From the remaining animals, data from 10 more animals (Non-stress vehicle miss, n = 3; non-stress drug, n = 3; stress vehicle, n = 1; stress drug, n = 3) were not used for statistical analyses because their cannulae placements were outside the boundaries of the PVN. The locations of the cannulae that were successful in hitting the PVN for animals in each experimental group are shown (see Figure 4). The majority of misplaced cannulae ended above or inside the third ventricle (n = 6). The remaining misplaced cannulae were located lateral to the PVN (n = 4).

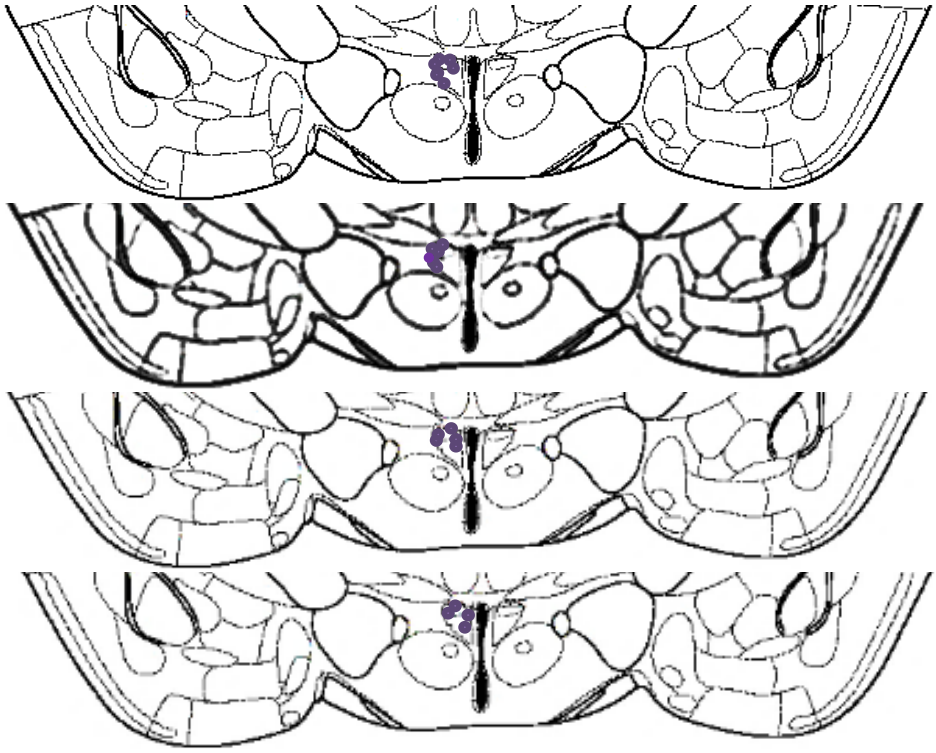

Figure 4: Location of cannulae placements in animals from each experimental group. Non-stress vehicle group, non-stress drug group, stress vehicle group, and stress drug group.

Chronic social stress increases chow consumption

During the baseline period, there were no significant differences in chow intake between the vehicle and drug treated mice. Following surgery, the mice were placed in a recovery period. Figure 5A depicts the mean and standard errors of chow intake during the recovery period with mice receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. Based on a one-way ANOVA, mice during the recovery period that received the drug infusion slightly increased their chow intake, [main effect of drug, $F_{(1,21)} = 3.245$, $p = .091$], although this did not reach statistical significance.

Figure 5B depicts the average chow intake in stress and non-stressed mice receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined that stressed mice increased their chow intake [main effect of stress, $F_{(1,21)} = 6.982$, $p < .05$] regardless of the drug infusion [main effect of drug, $F_{(1,21)} = 0$, $p > .05$] and there were no interactions, [interaction, $F_{(1,21)} = 3.378$, $p > .05$].

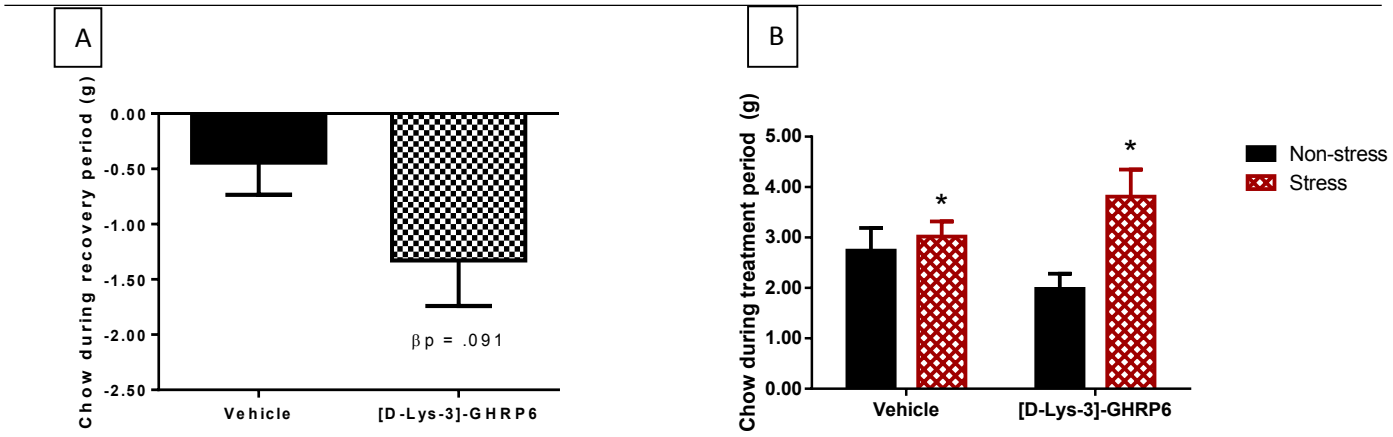

Figure 5: Standard laboratory chow consumption. A) Mean (±SEM) chow consumption calculating the difference between the end of baseline period and the end of the recovery period (g). B) Mean (±SEM) chow consumption during the treatment period (g). β $p = .091$ relative to vehicle controls. * $p < .05$ relative to non-stressed animals.

Chronic social stress decreases high fat diet consumption

During the baseline, there were no significant differences in high fat diet consumption between the vehicle and drug treated mice, the means for each group were .97 ± .17 g, .74 ± .19 g, 1.15 ± .09 g, 1.07 ± .22 g. Figure 6A depicts the mean and standard errors of high fat diet in animals receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN during the recovery period. A one-way ANOVA determined that there were no significant differences in high fat diet consumption between the drug and vehicle groups, [main effect of drug, $F_{(1,21)} = 2.557$, $p > .05$].

Figure 6B depicts the average high fat diet intake of stress and non-stressed mice receiving either the vehicle or ghrelin receptor antagonist infused within the PVN. A two-way ANOVA determined that stressed mice slightly decreased their high fat diet, [main effect of stress, $F_{(1,21)} = 3.293$, $p = .088$] although this did not reach significance. This effect was independent of the drug infusion, [main effect of drug, $F_{(1,21)} = .657$, $p > .05$] and there were no interactions, [interaction, $F_{(1,21)} = 2.643$, $p > .05$].

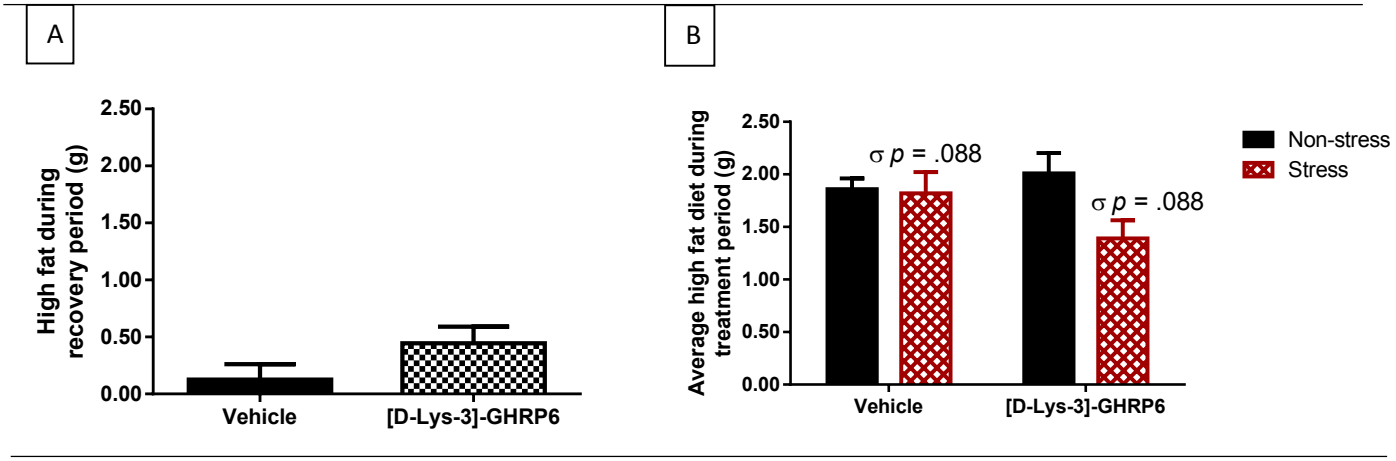

Figure 6: A) Mean (±SEM) high fat diet during the recovery period (g). B) Mean (± SEM) high fat diet during the treatment period (g). σ p = .088 relative to non-stressed animals.

Chronic social stress increases total daily caloric intake, primarily from calories from chow intake

Total caloric intake was calculated daily throughout the study through the addition of caloric content of standard laboratory chow and high fat diet. Caloric intake = [(Chow intake of 24 hours g) * (3.3 kcal)] + [(High fat diet 4 hours g) * (5.1 kcal)].

Figure 7A depicts the mean and standard errors of calories from chow intake in animals receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined that stressed animals increased their calories from chow, [main effect of treatment, $F_{(1,21)} = 6.881$, $p < .05$] and this was regardless of the drug infusion, [main effect of drug, $F_{(1,21)} = .001$, $p > .05$] with no interactions, [interaction, $F_{(1,21)} = 3.603$, $p < .05$].

Figure 7B depicts the mean and standard errors of calories from high fat diet intake in animals receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined that stressed animals increased high fat diet, [main effect of treatment, $F_{(1,21)} = 4.178$, $p = .057$] regardless of the drug infusion, [main effect of drug, $F_{(1,21)} = .982$, $p > .05$] and there were no interactions, [interaction, $F_{(1,21)} = 2.256$, $p > .05$].

Figure 7C depicts the mean and standard errors of average calories on stress and non-stressed mice delivering either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined that stressed animals increased their caloric intake, [main effect of treatment, $F_{(1,21)} = 8.490$, $p < .05$] and this was regardless of the drug infusion, [main effect of drug, $F_{(1,21)} = .024$, $p > .05$] with no interactions, [interaction, $F_{(1,21)} = .357$, $p < .05$].

Caloric intake = [(Chow intake of 24 hours g) * (3.3 kcal)] + [(High fat diet 4 hours g) * (5.1 kcal)]

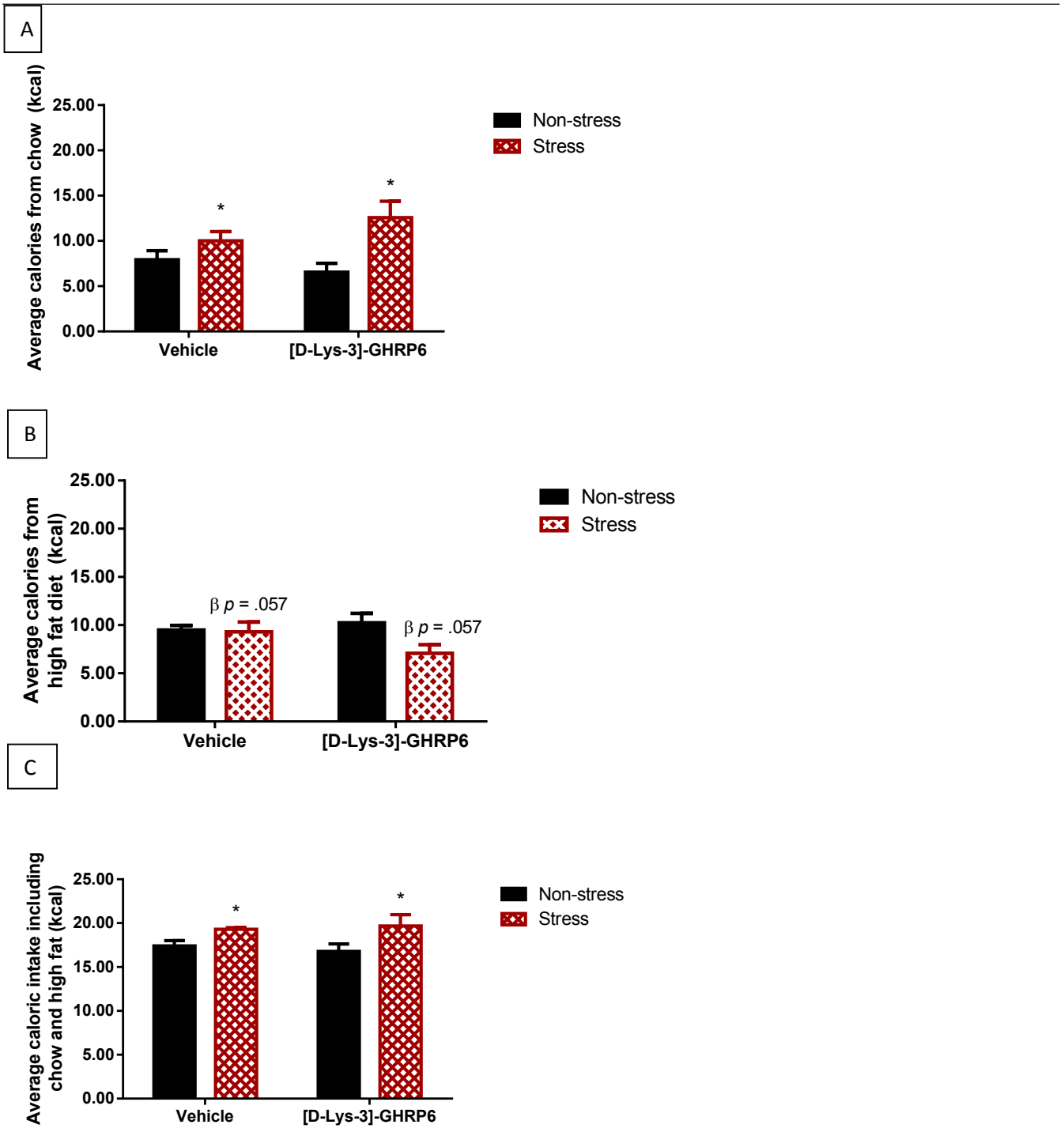

Figure 7: A) Mean (± SEM) calories taken from chow intake (kcal). B) Mean (±SEM) calories taken from high fat diet intake (kcal). C) Mean (±SEM) calories taken from chow and high fat diet (kcal). * $p < .05$ relative to non-stressed animals. β $p = .057$ relative to non-stressed animals.

Chronic social stress decreases overall body weight and specifically retroperitoneal adipose tissue, but not BAT

During the baseline, there were no significant differences in the body weight between the vehicle and the drug groups, means for each group were 27.33 ± 1.04 g, 27.66 ± .80 g, 27.71 ± 1.25 g, 28.46 ± 1.00 g. Figure 8A depicts the mean and standard errors of body weight in animals receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A one-way ANOVA determined there were no significant differences in body weight between the vehicle and drug infusions during the recovery period [main effect of drug, $F_{(1,21)} = .510$, $p > .05$].

Figure 8B depicts the mean and standard errors of body weight in stress and non-stressed mice receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined there were significant differences in body weight between the drug groups and between treatment groups, [main effect of drug, $F_{(1,21)} = .105$, $p > .05$; main effect of stress, $F_{(1,21)} = .282$, $p > .05$]. Analysis also indicated there was a near-significant interaction between the drug and stress groups, [interaction, $F_{(1,21)} = 4.208$, $p = .056$]. LSD post-hoc analysis indicated that stressed mice receiving the vehicle slightly decreased their body weight compared with non-stressed vehicle controls, $p = .076$. Interestingly, this effect was not seen in antagonists treated mice ($p > 0.05$).

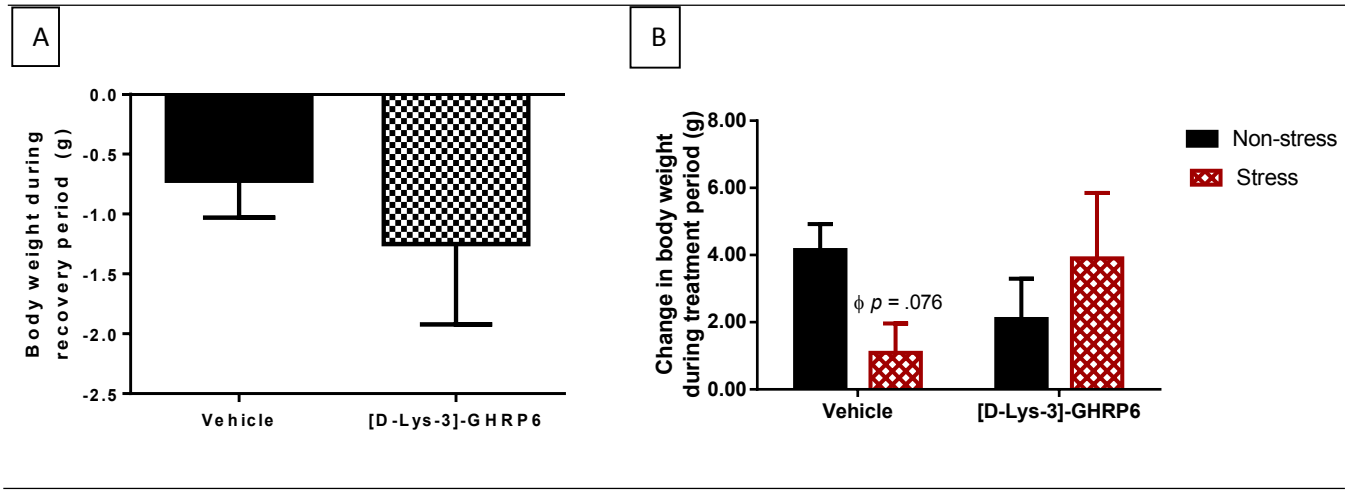

Figure 8: A) Mean (± SEM) body weight during the recovery period (g). B) Change in body weight from the average of the baseline period and the average of treatment period (g). ϕ p = .076 relative to the vehicle non-stressed animals.

Figure 9 depicts the mean and standard errors of subcutaneous, retroperitoneal, perigonadal, and BAT in stress and non-stressed mice receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN.

Figure 9A depicts the mean and standard errors of subcutaneous adipose tissue receiving either the vehicle and drug infusion with stress and non-stress groups. A two-way ANOVA determined that stressed animals showed slightly decreased subcutaneous adipose tissue, [main effect of stress, $F_{(1,21)} = 3.086$, $p = .096$] regardless of the drug infusion, [main effect of drug, $F_{(1,21)} = .402$, $p > .05$] and there were no interactions, [interaction, $F_{(1,21)} = .603$, $p > .05$].

Figure 9B depicts the mean and standard errors of retroperitoneal adipose tissue delivering either the vehicle and drug infusion with stress and non-stress groups. Analysis determined that stressed animals decreased retroperitoneal adipose tissue, [main effect of stress, $F_{(1,21)} = 5.335$, $p < .05$] with no significant differences in retroperitoneal tissue between the drug groups, [main effect of drug, $F_{(1,21)} = .097$, $p > .05$] with no interactions between the stress and drug groups, [interaction, $F_{(1,21)} = .051$, $p > .05$].

Figure 9C depicts the mean and standard errors of perigonadal adipose tissue. A two-way ANOVA showed no significant differences in perigonadal tissue in stress and non-stressed mice receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN, [main effect of stress, $F_{(1,21)} = 2.186$, $p > .05$] and analysis showed no significant differences in perigonadal adipose tissue between the drug groups, [main effect of drug, $F_{(1,21)} = .435$, $p > .05$] and there were no interactions between the stress and drug groups, [interaction, $F_{(1,21)} = .688$, $p > .05$].

Figure 9D depicts the mean and standard errors of BAT. A two-way ANOVA failed to detect significant differences in BAT in stress and non-stressed mice receiving either the vehicle

or ghrelin receptor antagonist infusion within the PVN, [main effect of stress, $F_{(1,21)} = .353$, $p > .05$] and there were no significant differences in BAT between the drug groups, [main effect of drug, $F_{(1,21)} = .320$, $p > .05$] and there were no significant interactions between the treatment and drug groups, [interaction, $F_{(1,21)} = .010$, $p > .05$].

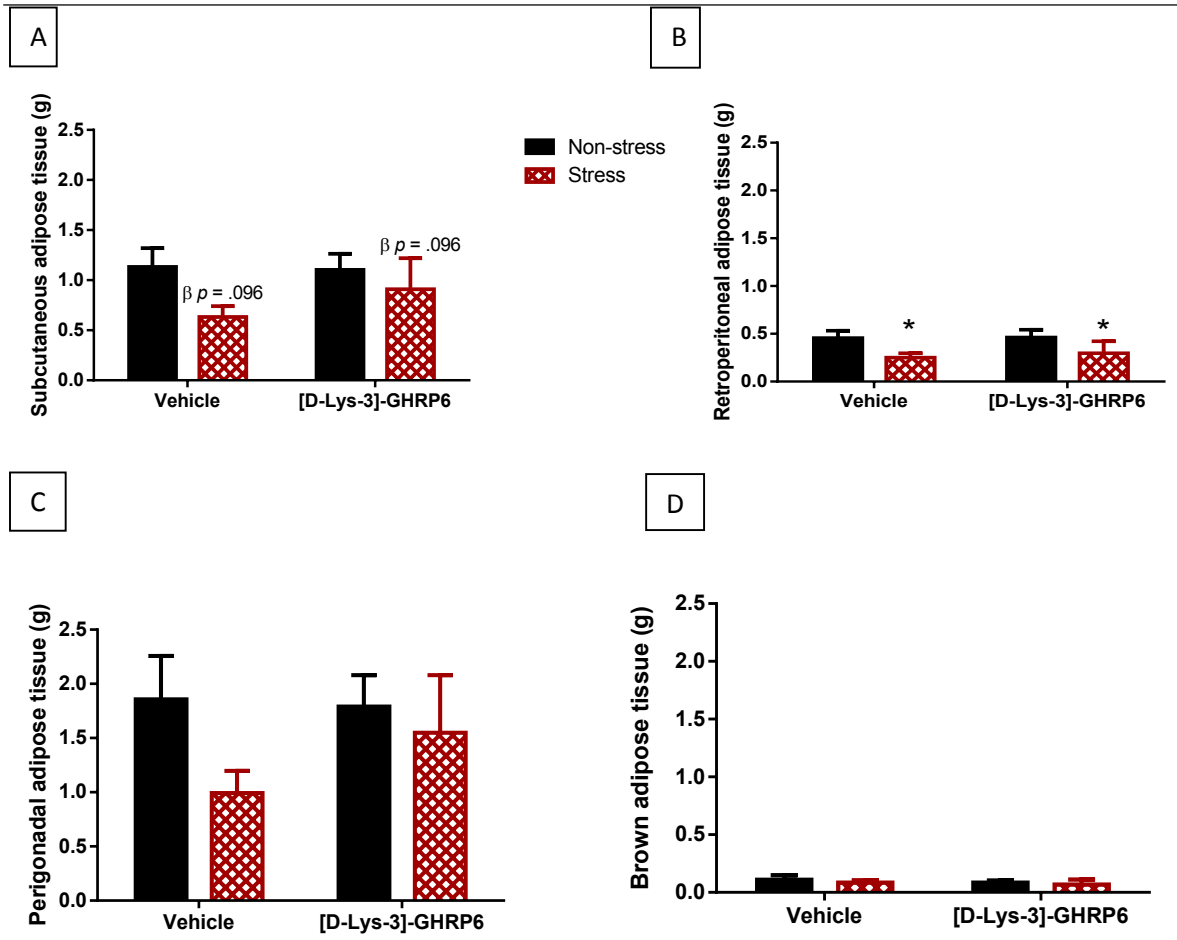

Figure 9: Various adipose tissue locations. A) Mean (±SEM) subcutaneous adipose tissue (g). B) Mean (±SEM) retroperitoneal adipose tissue (g). C) Mean (±SEM) perigonadal adipose tissue (g). D) Mean (±SEM) BAT (g). β $p = .096$ relative to the non-stress animals. * $p < .05$ relative to the non-stress animals.

Chronic social stress decreases UCP-1 and β3-adrenergic receptor and slightly decreased HSL mRNA expression within BAT

Figure 10A depicts the mean and standard errors of UCP-1 mRNA expression within BAT collapsing the data in stress and non-stressed groups with animals receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA showed that stressed animals had lower UCP-1 mRNA expression within BAT, [main effect of stress, $F_{(1,21)} = 4.715$, $p < .05$]. This effect was independent of the drug infusions, [main effect of drug, $F_{(1,21)} = .039$, $p > .05$] and there were no interactions between the drug and the treatment groups [interaction, $F_{(1,21)} = 1.106$, $p > .05$].

Figure 10B depicts the mean and standard errors of β3-adrenergic receptor mRNA expression within BAT collapsing the data in stress and non-stressed groups with animals receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined that stressed animals showed decreased β3-adrenergic receptor within BAT, [main effect of stress, $F_{(1,21)} = 4.695$, $p < .05$] regardless of the drug infusion, [main effect of drug, $F_{(1,21)} = .734$, $p > .05$] and there were no interactions between the treatment and drug groups, [interaction, $F_{(1,21)} = 2.599$, $p > .05$].

Figure 10C depicts the mean and standard errors of hormone sensitive lipase (HSL) mRNA expression within BAT collapsing the data in stress and non-stressed groups with animals receiving with either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined that stressed animals decreased HSL mRNA expression within BAT, [main effect in stress, $F_{(1,21)} = 3.179$, $p = .091$] although this effect did not reach statistical significance. This effect was regardless of the drug infusion, [main effect of drug, $F_{(1,21)} = .118$, $p > .05$] and there were no interactions between the treatment and the drug groups, [interaction, $F_{(1,21)} = .667$, $p > .05$].

Ghrelin signaling within the PVN and chronic social stress are independent of PGC-1α activity within BAT

Figure 10D depicts the mean and standard errors of PGC-1α mRNA expression within BAT collapsing the data in stress and non-stressed groups with animals receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined that there were no significant differences in PGC-1α mRNA expression within BAT with animals receiving the drug infusion, [main effect of drug, $F_{(1,21)} = 2.042$, $p > .05$] and no significant treatment effects between groups, [main effect of stress, $F_{(1,21)} = 1.212$, $p > .05$] and no significant interactions between the drug and treatment groups [interaction, $F_{(1,21)} = .231$, $p > .05$].

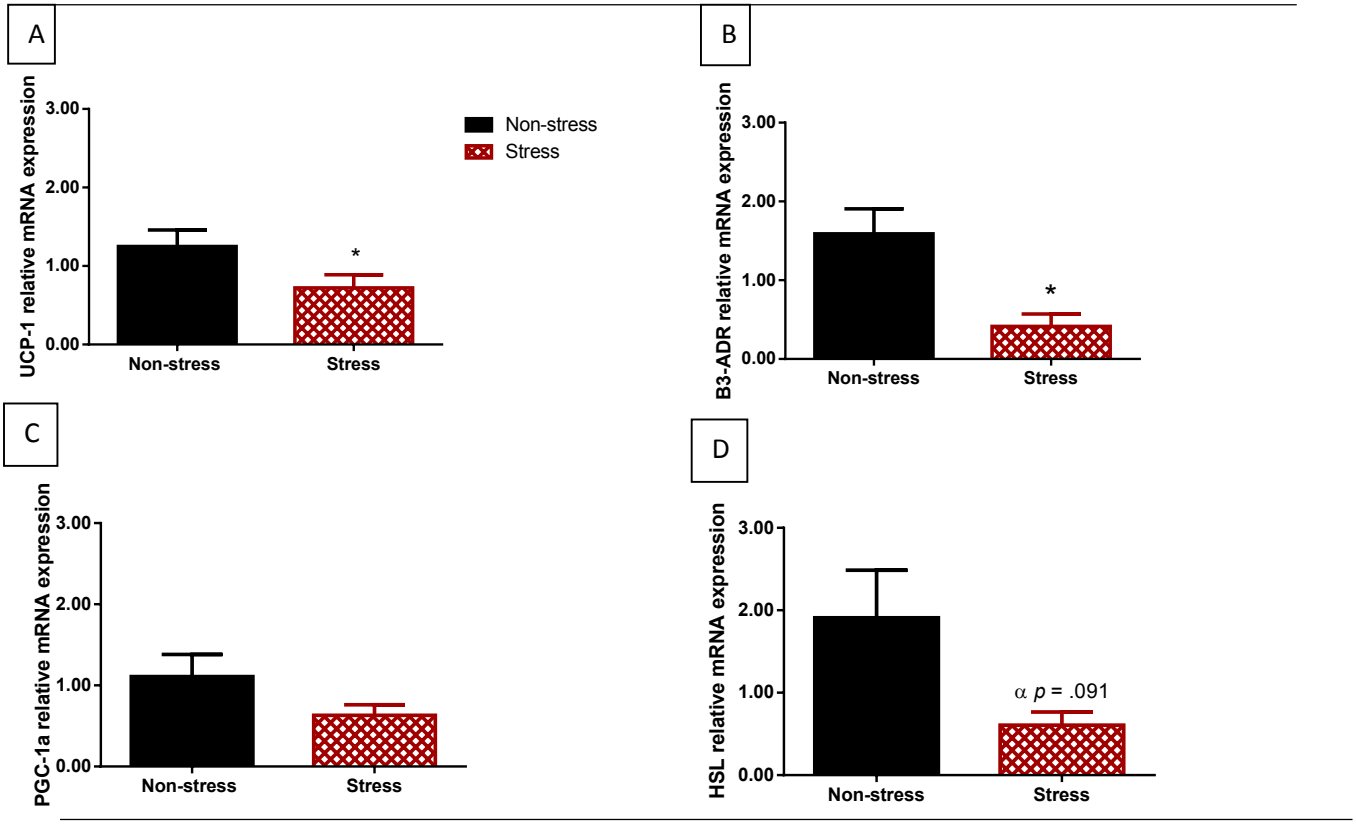

Figure 10: A) Mean (± SEM) relative mRNA expression of UCP-1 within BAT. B) Mean (± SEM) relative mRNA expression of β3-Adrenergic receptor within BAT. C) Mean (± SEM) relative mRNA expression of PGC-1α within BAT. D) Mean (± SEM) relative mRNA expression of HSL within BAT. * $p < .05$ relative to non-stressed animals. α $p = .091$ relative to non-stressed animals.

Non-stressed animals receiving the drug infusions showed decreased plasma epinephrine levels

Figure 11A depicts the mean and standard errors of plasma EP in stress and non-stressed mice receiving either the vehicle or ghrelin receptor antagonist infusion within the PVN. A two-way ANOVA determined there was no significant differences in plasma EP between drug groups, [main effect of drug, $F_{(1,21)} = .03$, $p > .05$] and no significant differences in plasma EP between stress groups, [main effect of stress, $F_{(1,21)} = .694$, $p > .05$]. Analysis did indicate there was a significant interaction between the drug and treatment groups, [interaction, $F_{(1,21)} = 10.451$, $p < .05$]. LSD post-hoc analysis showed non-stressed animals receiving the drug infusion had decreased plasma EP levels compared to the vehicle non-stressed controls, $p < .05$. Our analyses also indicated that non-stressed animals receiving the drug infusions had significantly lower plasma EP levels than the stressed drug group, $p < .05$. In addition, the vehicle stress group showed significantly lower plasma EP levels than the vehicle controls, $p < .05$.

Chronic social stressed animals receiving the drug infusion showed higher plasma norepinephrine levels

Figure 11B depicts the means and standard errors of plasma NE levels in stress and non-stressed mice receiving either vehicle or ghrelin receptor antagonists infused into the PVN. A two-way ANOVA determined that stressed animals had higher plasma NE concentrations than non-stressed mice although this difference failed to reach statistical significance [main effect of stress, $F_{(1,21)} = 3.696$, $p = .074$]. Similarly, mice receiving the ghrelin receptor antagonist by the PVN had higher plasma NE levels compared to vehicle treated mice but this effect also failed to attain statistical significance, [main effect of drug, $F_{(1,21)} = 3.754$, $p = .072$]. Finally, there was a significant interaction effect [interaction, $F_{(1,21)} = 4.760$, $p < .05$], and LSD post-hoc analyses showed that stressed mice receiving drug infusions had significantly higher plasma NE levels compared to mice in the remaining groups, $p < .05$.

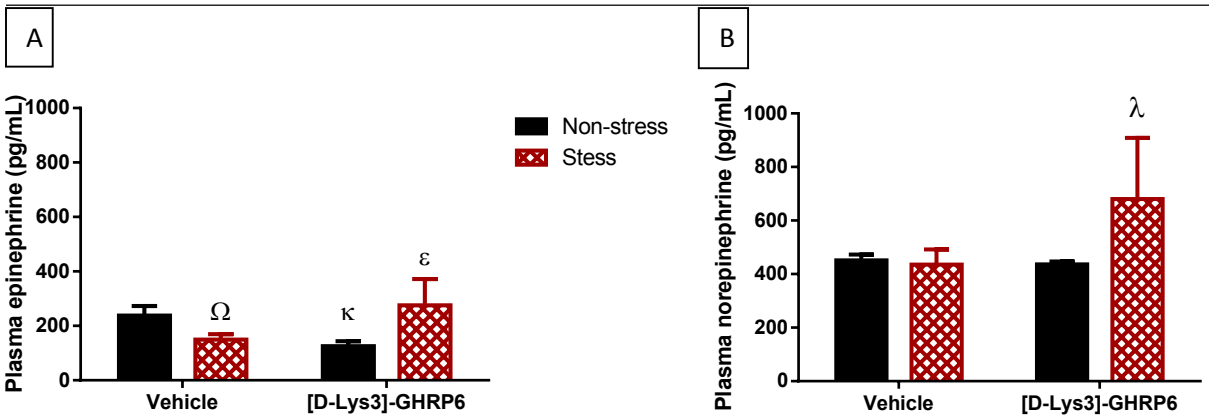

Figure 11: A) Mean (± SEM) plasma adrenaline. B) Mean (± SEM) plasma epinephrine. Ω $p <$ .05 relative to non-stress vehicle controls. κ $p <$ .05 relative to non-stressed vehicle controls. ε $p <$ .05 relative to non-stressed animals receiving drug infusions. λ $p <$ .05 relative to the remaining groups.

Non-stressed animals receiving the drug infusion showed increased MHPG levels within BAT

Figure 12A depicts the means and standard errors of MHPG levels within BAT in stress and non-stressed mice receiving either vehicle or ghrelin receptor antagonists infused into the PVN. A two-way ANOVA determined that there were no significant differences in MHPG levels within BAT between drug groups, [main effect of drug, $F_{(1,21)} = 2.541$, $p > .05$] and there were no significant differences in MHPG levels between treatment groups, [main effect of stress, $F_{(1,21)} = .242$, $p > .05$]. Analysis did indicate a near-significant interaction effect between the drug and stress group, [interaction, $F_{(1,21)} = 4.124$, $p = .062$]. LSD post-hoc analyses determined that non-stressed animals receiving the drug infusion had significantly higher MHPG levels within BAT compared with the vehicle controls, $p < .05$. In addition, we found stressed animals receiving the drug infusions had slightly lowered MHPG levels than non-stressed animals receiving the drug infusion, but this effect did not reach statistical significance, $p = .096$.

Ghrelin signaling within the PVN or chronic social stress has no effect on norepinephrine or epinephrine levels with BAT

Figure 12B depicts the means and standard errors of NE within BAT in stress and non-stressed mice receiving either vehicle or ghrelin receptor antagonists infused into the PVN. A two-way ANOVA determined there were no significant differences in NE within BAT between the drug groups, [main effect of drug, $F_{(1,21)} = .354$, $p > .05$]. There were no treatment effects from NE levels within BAT, [main effect of stress, $F_{(1,21)} = .477$, $p > .05$]. In addition, there was no significant interaction effect between the drug and the stress groups, [interaction effect, $F_{(1,21)} = .138$, $p > .05$].

Figure 12C depicts the means and standard errors of EP within BAT in stress and non-stressed mice receiving either vehicle or ghrelin receptor antagonists infused into the PVN. A

two-way ANOVA determined there were no significant differences in EP within BAT between the drug groups, [main effect of drug, $F_{(1,21)} = 1.201$, $p > .05$]. There were no significant differences in EP within BAT between the treatment groups, [main effect of stress, $F_{(1,21)} = .046$, $p > .05$]. There were also no interactions between the drug and the treatment effect, [interaction, $F_{(1,21)} = .086$, $p > .05$].

Non-stressed animals receiving the drug infusion showed increased MHPG/NE ratios within BAT

Figure 12D depicts the means and standard errors of MHPG/NE within BAT in stress and non-stressed mice receiving either vehicle or ghrelin receptor antagonists infused by the PVN. A two-way ANOVA determined there were no significant differences in MHPG/NE between the treatment groups, [main effect of stress, $F_{(1,21)} = .349$, $p > .05$] and no significant differences between the drug groups, [main effect of drug, $F_{(1,21)} = 2.187$, $p > .05$]. Analyses did indicate a near-significant interaction between the drug and stress group, [interaction, $F_{(1,21)} = 4.318$, $p = .057$]. LSD post-hoc analysis determined that non-stressed animals receiving the drug infusion showed significantly higher MHPG/NE ratio relative to vehicle controls, $p < .05$. In addition, there was a higher MHPG/NE ratio in drug non-stressed animals compared to drug stress animals, but this effect did not reach statistical significance, $p = .08$.

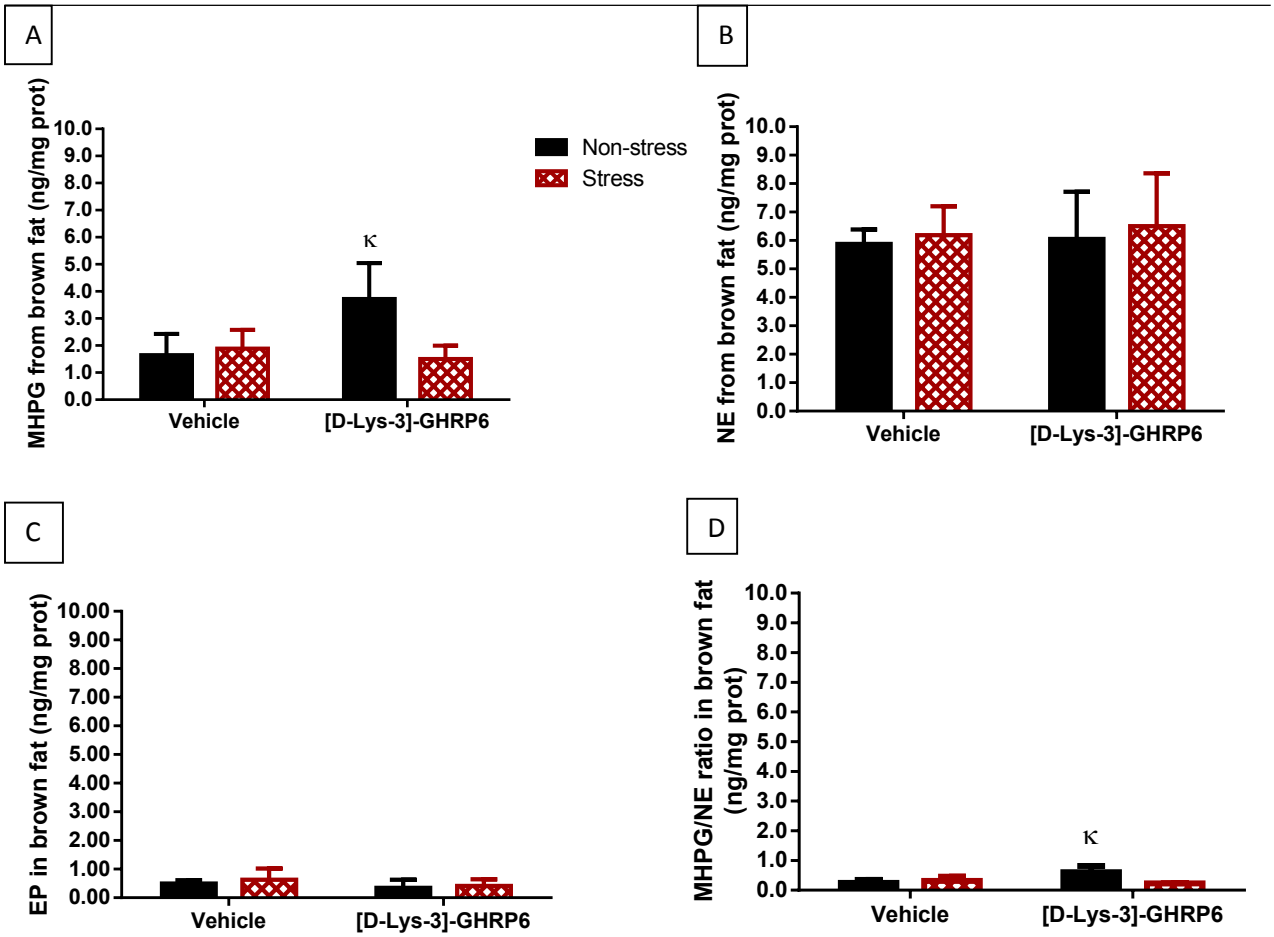

Figure 12: A) Mean (± SEM) MHPG in BAT. B) Mean (± SEM) NE within BAT. C) Mean (± SEM) epinephrine in BAT. D) Mean (± SEM) MHPG/NE ratio in brown fat. κ $p < .05$ relative to non-stress vehicle.

Discussion

In the current thesis, we hypothesized that psychological stress modulates UCP-1 expression in BAT and that this is mediated through the activation of ghrelin receptors in the PVN. This hypothesis was generated given that ghrelin is secreted during stress and produces changes in food intake and energy expenditure thereby facilitating the utilization of glucose over fat as a source of fuel (Patterson *et al.,* 2013b). It was also based on data suggesting that ghrelin modulates the activity of BAT, in particular UCP-1 expression, possibly through central mechanisms (Yasuda *et al.,* 2003). Results from the current study partially supported this hypothesis.

As expected, chronic social defeat resulted in a number of behavioral and metabolic alterations. As shown in previous work from our lab (Patterson *et al.,* 2013a; Patterson *et al.,* 2013b), chronic social defeat resulted in higher caloric consumption and this is similar to our study, as seen in Figure 7C. This effect was not altered by the delivery of the GHSR antagonist into the PVN. The caloric intake was primarily due to the increase of chow, showing animals had a preference to replenish their depleting carbohydrate stores. Interestingly, ghrelin receptor blockade in the PVN resulted in a slight increase in high fat diet before the onset of the stress protocol, as seen in Figure 6A. We have previously recorded this effect, where animals with the GHSR antagonist had a preference towards high fat diet consumption (Patterson *et al.,* 2013a). However, we did not see this effect throughout the treatment period, when in fact stressed animals consumed less of the high fat diet compared to non-stressed mice, regardless of the drug infusion (Figure 6B). This suggests that ghrelin receptors in the PVN may promote the preference for carbohydrate rich diets yet during a metabolic challenge, other factors may have contributed to the change in diet preference.

Ghrelin's effect on body weight during the stress response shows a decrease in adipose tissue expenditure and this effect is mediated through GHSR. For instance, GHSR-KO mice have depleted adipose tissue during stress when compared to stressed WT mice (Patterson *et al.,* 2013b). In the present study, stress seemed to have an overall lipolytic effect in subcutaneous and retroperitoneal fat depots, as seen in Figure 9A and 9B, respectively. Figure 9D shows there were no effects of the stressor or drug infusion on total BAT mass. This may be due to the amount of BAT present at the time. Some BAT mass was used for HPLC and the overall BAT mass was low. In addition, no differences in BAT mass could also be due to their age, we used young mice while another study has mentioned having difficulty showing changes in expression in BAT along with BAT mass in younger mice (Lin *et al.,* 2011). Our data would suggest that if ghrelin protects brown fat from being utilized as a source of energy during stress, this is not mediated by ghrelin acting on the PVN.

This was further confirmed by our PCR data. While chronic social stress decreases BAT activation as reflected by a decrease in the expression of transcripts associated with BAT activity (i.e. the expression of β3 adrenergic receptors, HSL, and UCP-1), the blockade of the GHSR at the level of the PVN had no obvious effects in influencing these changes, as seen in Figure 10. This again points to areas other than the PVN as potential targets of ghrelin during stress to modulate the activity of BAT. One of these could be brown adipocytes themselves, as they express the GHSR (Lin *et al.,* 2011). A peripheral approach may be beneficial, as Lin and colleagues (2011) found that GHSR KO mice had increased thermogenic capacity compared to WT mice (Lin *et al.,* 2011).

Alternatively, ghrelin released during stressed may target other hypothalamic regions also associated with stress and connected to adipose stores that together increase the activity of BAT.

One of these regions is the dorsomedial hypothalamus (DMH), a region that also expresses the GHSR. A recent paper shows that psychological stress stimulates neurons within the DMH and these cells modulate BAT activity through the midbrain medullary raphe (Kataoka *et al.,* 2014). Therefore, ghrelin may act at these areas to regulate BAT activity, a hypothesis that requires further testing.

Interestingly, ghrelin receptor blockade altered noradrenergic turnover in BAT, as seen in Figure 12D. While there were no differences in EP or NE within BAT samples collected from mice in the different groups, MHPG levels were significantly elevated in mice treated with the ghrelin receptor antagonist, indicating increased noradrenergic utilization in BAT from these mice, shown in Figure 12A. This effect, however, was no longer evident in stressed mice receiving the GHSR antagonist. The effects of the antagonist seen here support the notion that ghrelin acts on the PVN to decrease noradrenergic tone on BAT. Blockade of the receptor in stressed mice may not yield increased utilization in BAT given that β3 adrenergic receptors are decreased in stressed mice, in spite of equal amounts of NE, therefore less utilization may occur. It is unclear if the levels of MHPG are elevated because of increased NE turnover or simply because more reuptake is occurring. Nevertheless, the elevated MHPG levels did not have an effect on BAT thermogenic expression, as GHSR treated animals did not show differentially altered expression of BAT related genes.

Our data showed that chronic stress leads to sympathetic alterations and some of these are countered by ghrelin receptor blockade in the PVN. For instance, plasma EP concentrations were reduced in chronically stressed mice, shown in Figure 11A, an effect that may reflect a compensatory mechanism. Previous data from our lab using the same drug within the PVN indicated that stressed animals showed higher plasma cortisol levels (Patterson *et al.,* 2013a). We

previously suggested that ghrelin may be important in the adaptive mechanisms that decrease cortisol levels during chronic stress (Patterson *et al.,* 2013a). This complements our findings that stressed animals receiving the drug infusion showed elevated plasma EP and NE levels, as seen in Figure 11A and 11B respectively. Therefore, with animals receiving the drug infusion, there is an elevated cortisol response and this is substantiated with the elevated plasma EP and NE levels in the stressed group. This compensatory mechanism would be set in place to prevent over secretion of EP and NE during chronic stress. Stressed mice receiving the antagonist, however, would get the additive effect of the stress and the drug, and would not be able to suppress the catecholaminergic response. In all, these data seem to point towards an inhibitory effect of ghrelin on catecholamine release from the adrenal medulla during the stress response and that is generated by ghrelin acting on the PVN.

Conclusion

In all, our data provide evidence suggesting that ghrelin receptors in the PVN play a role in modulating sympathetic responses, which may affect the sympathomedullary responses to chronic social stressors. These responses may not include the activity of BAT, as shown by evidence from the present study. Given that the activity of BAT has also been associated with the stimulation of hypothalamic regions like the DMH (Kataoka *et al.,* 2014) and VMH (Amir, 1990) and the GHSR is also present in these regions, one could suggest that the effects of ghrelin on BAT are not mediated by the PVN but perhaps mediated by the action of ghrelin on the DMH and/or VMH. Alternatively, the effects of ghrelin on BAT may be produced directly at the level of the brown adipocyte, where the GHSR is present. Clearly, more studies are required to make an assessment of the contributions of all of these regions and to shed some light towards ghrelin and the activity of BAT under normal and stressed conditions. Ultimately, these research questions will also provide more knowledge on hypothalamic regulation of energy expenditure,

the hormones that influence these processes, and potentially unveil therapeutic targets to combat metabolic related disorders like obesity, cardiovascular disease, and type II diabetes.

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