The motivation to obtain highly palatable food is enhanced by ghrelin in the ventral tegmental area

by

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Abstract

The current studies examined the effects of chronic ghrelin administration in the ventral tegmental area (VTA) on 1) ad libitum food intake and bodyweight; 2) macronutrient preference; and 3) motivation to obtain highly palatable chocolate pellets. Male rats were implanted with 14-day osmotic mini-pumps delivering either saline, 3nM of ghrelin, or 10nM of ghrelin/day directly into the VTA. Ghrelin dose-dependently increased intake of regular chow (RC) in the home cage, as well as body weight gain across the infusion period ($p < .05$). Next, we implanted rats with mini-pumps delivering saline, 10 nM of ghrelin or a 200nM of a ghrelin receptor antagonist ([Lys-3]-GHRP-6), examining preference for three macronutrient diets high in fat, protein, or carbohydrates. [Lys-3]-GHRP-6 significantly reduced caloric intake of high-fat chow ($p<.05$) and decreased body weight ($p< .05$), relative to ghrelin treated rats. A final group of rats were tested in an operant chamber under a progressive ratio schedule to obtain chocolate pellets. By Day 14, [Lys-3]-GHRP-6 treated rats exhibited the lowest breakpoints, whereas ghrelin treated rats displayed an increase in effort expended to obtain food reward ($p< .05$). Together, these results suggest that ghrelin modulates hedonic aspects of feeding, increasing preference for and motivation to obtain highly-palatable food.

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List of Abbreviations

6-OHDA- 6-hydroxydopamine

AgRP- agouti-related peptide

ARC- arcuate nucleus of the hypothalamus

BBB- blood brain barrier

BMI- body mass index

BP- breakpoint

CART- cocaine-amphetamine related transcript

CNS- central nervous system

CPP- conditioned place preference

CR- caloric restriction

DA- dopamine

[D-Lys3]-GHRP-6- [D-Lys3]-Growth Hormone Releasing Peptide-6 (GHRP-6)

FR- fixed ratio

Fra1- fos-related antigen 1

Fra2- fos-related antigen 2

GHSR- growth hormone secretagogue receptor (1a OR 1b)

GPCR- g-protein coupled receptor

HFD- high-fat diet

ICV- intracerebroventricular

LH- lateral hypothalamus

mRNA- messenger ribonucleic acid

NAc- nucleus accumbens

NPY- neuropeptide Y

POMC- proopiomelanocortin

PFC- prefrontal cortex

PR- progressive ratio

VMH- ventromedial hypothalamus

VTA- ventral tegmental area

WHO- World Health Organization

The motivation to obtain highly palatable food is enhanced by ghrelin in the ventral tegmental area

The obesity epidemic

The past few decades have seen a steady rise in the prevalence of obesity, with a corresponding increase in research efforts attempting to elucidate both the genetic and environmental influences on its development. Obesity is defined as an excess amount of body fat; a positive energy balance that occurs when increases in food intake exceed the amount of energy expended. The most common index used to recognize obesity is the body mass index (BMI), a ratio of body weight to height (kg/m^2). The World Heatlh Organization (WHO) posits that an individual with a BMI between 25 and 29 is considered overweight, with a score of 30 or above being defined as obese (WHO, 2000). BMI does not take into account the distribution of body fat however, and the waist circumference of an individual is thought to be a more accurate predictor of health risk. Using this measure, obesity is defined as a waist circumference of greater than 102 cm in men and greater than 88 cm in women (Squire et al., 2008).

The global obesity epidemic is thought to result from a combination of factors such as genetic susceptibility, an abundance of energy-dense foods, as well as an increase in the prevalence of sedentary lifestyles (Kopelman, 2000). Such an 'obesogenic environment' can lead to an increase in fat mass, which can further lead to a host of medical problems including, but not limited to: heart disease, hypertension, type 2 diabetes, stroke, cancer, and both gastrointestinal and reproductive dysfunction (Lawrence & Kopelman, 2004). A better understanding of the physiological systems responsible for maintaining energy homeostasis and the conditions in which it can be chronically taxed or become abhorrent is necessary to discover what types of therapeutic intervention may ameliorate symptoms of obesity.

Central regulation of appetite and body weight

Feeding is a necessary component of an organism's survival and reproductive success. In mammals, food intake is a regulatory process ensuring that the body receives adequate energy (i.e. glucose) to meet its physiological demands. The prevailing theory regarding the regulation of food intake involves *caloric homeostasis*, during which hunger is initiated by a gradual reduction of satiety signals generated by a previous feeding bout (i.e. a meal) (Squire et al., 2008). However, other variables such as learning, social factors and various environmental stimuli are also thought to influence ingestive behaviour. Mammalian species have evolved several neural mechanisms to facilitate the acquisition of an abundance of nutritive food sources.

Although the basic tenet of energy balance appears deceivingly simple (i.e. a healthy balance between energy consumed and expended), the physiological control of energy balance and appetite is both complex and multifaceted. Homeostatic mechanisms provide the organism with a means of sensing the nutritional content of food and monitoring energy stores, providing feedback to increase or decrease energy expenditure as necessary. However, despite the abundance of research being conducted in this field, the underlying mechanisms of energy regulation are yet to be fully understood.

Early lesion studies in rats demonstrated the importance of the hypothalamus in regulating food intake (Hetherington, 1940; Hetherington & Ranson, 1942). Briefly, lateral hypothalamic (LH) lesioned rats showed hypophagia, or a significant reduction in feeding, whereas rats that received lesions to the ventromedial hypothalamus (VMH) displayed hyperphagia and obesity. Electrical stimulation studies further supported these findings, showing that stimulation of the LH and VMH produced obesity and anorexia, respectively (Coons & Cruce, 1968; Valenstein et al., 1968). Taken together, this research led to a 'dual center'

hypothesis suggesting that the LH was responsible for orexigenic signaling (i.e. appetite stimulating) and that, conversely, the VMH provided satiety signals (Elmquist, Elias & Saper, 1999).

This simple hypothesis lacked the ability to address questions regarding the ability of these regions (and possibly others) to influence the short term regulation of meal patterns, the long term maintenance of body weight, as well as the influence of motivation on feeding behaviour. Further research efforts soon revealed that the aforementioned discrete hypothalamic nuclei did not serve simply as hunger and satiety centers. Destruction of select dopaminergic fiber pathways *connecting* various hypothalamic regions, using 6-hydroxydopamine (6-OHDA) lesions, were shown to be sufficient to induce similar pathologies to those seen in the lesioned mice (Zigmond & Stricker, 1972). These results suggested an important role for the neurochemical regulation of food intake and appetite.

Peptide Regulation of Energy Balance

With the discovery that food intake is associated with the maintenance of body weight, the ability of adipose tissue to generate signals that could regulate food intake became a popular area of inquiry. It was soon discovered that after a period of food deprivation and reduction in body weight, animals ate a larger meal to compensate until their body weight returned to its "set point" (Harris & Martin, 1983). On the other hand, after force feeding and weight gain, smaller meals were consumed by the animal until the weight was lost. These compensatory mechanisms suggest that a regulatory feedback system is in place allowing adipose tissue to relay information to central regions indicating when to obtain more nutrients, or conversely, when to stop accumulating energy.

The modulation of feeding behaviour via adipose tissue is thought to be controlled largely by circulating hormones or peptides. Numerous peripheral signals of adiposity act within the central nervous system to influence feeding and body weight regulation. Leptin is one such hormone that acts as a negative feedback signal to the brain resulting in the inhibition of feeding. Synthesized by white adipose tissue in the periphery, leptin can cross the blood-brain barrier (BBB) and acts mainly on the hypothalamic arcuate nucleus (ARC) to suppress feeding. Leptin receptors in the ARC are found on two subpopulations of neurons that express pro-opiomelanocortin (POMC) and cocaine-amphetamine related transcript (CART). Activation of leptin receptors on these cells causes an inhibition of neuropeptide Y (NPY) /agouti-related peptide (AgRP) neurons which typically serve to enhance appetite. Mice that lack the gene coding for leptin (*ob/ob*) become hyperphagic and obese, whereas direct infusion of leptin causes the knock-out mice to reduce their food intake (Zhang et al., 1994). A more recently discovered peptide, ghrelin, has been shown to have opposing effects on appetite and body weight.

Ghrelin

Ghrelin is a 28 amino-acid peptide that is produced primarily in the X/A-like endocrine cells in the oxyntic glands of the stomach (Kojima et al., 1999; Date et al., 2000). The functional properties of ghrelin are reliant on the post-translational addition of an octanoyl group in the position of the third serine residue, a process referred to as acetylation (Kojima et al., 1999). The second form of ghrelin, the non-acetylated form, is known as des-acyl ghrelin, and constitutes a greater percentage of the ghrelin found in circulation (Guan et al., 1997). The physiological effects of des-acyl ghrelin are thought to differ from octanoylated ghrelin, although the formers role has not yet been fully elucidated.

Ghrelin was discovered as the endogenous ligand to the growth hormone secretagogue receptor (GHSR) in 1999 (Kojima et al., 1999). The metabotropic GHSR is a 7-transmembrane member of the g-protein coupled receptor (GPCR) family, which results in the activation of intracellular signal transduction pathways. GHSR messenger ribonucleic acid (mRNA) is expressed widely in the periphery, as well as in the central nervous system (Guan et al., 1997; Zigman et al., 2006). Two GHSR receptor subtypes have been identified, GHSR-1A and GHSR-1B. The GHSR 1A receptor has been well characterized, both structurally and functionally, and appears to be the most physiologically relevant in feeding and metabolic processes. The GHSR-1B receptor is a splice variant of the GHSR-1A and its functional significance has not been discovered to date (Howard et al., 1996).

The ability of ghrelin to cross the blood-brain-barrier (Banks, Tschop, Robinson & Heiman, 2002) promotes its action on the ARC, contributing to homeostatic mechanisms including the initiation of a meal and a reduction in the utilization of fat as an energy source (Tschop et al., 2000). Within the ARC, ghrelin activates orexigenic NPY/AgRP neurons, both of which express ghrelin receptors (Willesen et al., 1999; Cowley et al., 2003). As mentioned previously, these neurons work in tandem with the POMC/CART neurons typically stimulated by leptin, and together, they help regulate a healthy energy balance. Intracerebroventricular (ICV) ghrelin administration increases the expression of the immediate early gene cFOS, a marker of neuronal activiation, in the ARC as well as a number of additional hypothalamic regions (Scott, McDade, & Luckman, 2007).

Functional properties of ghrelin

Ghrelin has been shown to stimulate the release of growth hormone when administered to both rodents and humans (Date et al., 2000; Kojima et al., 1999). It is also the only known

orexigenic peptide that stimulates feeding when given peripherally (Hosoda, Kojima & Kangawa, 2006). Many studies have shown that ghrelin increases food intake and body weight gain when administered systemically, via acute or chronic injections in rats (Lawrence et al., 2002; Tschop et al., 2000; Wren et al., 2000; Wren et al., 2001). Further, these effects appear to be due to increased meal frequency rather than to increase in meal size (Faulconbridge et al., 2003). Circulating ghrelin levels are heightened during times of negative energy balance, in both short-term (e.g. during a fast) and long-term (e.g. anorexia nervosa) conditions (Tohsinai et al., 2001; Nagaya et al., 2001). Conversely, plasma ghrelin is decreased during times of abundant energy, such as immediately following a meal, as well as in chronic conditions such as obesity (Tschop et al., 2001). In humans, ghrelin levels show daily rhythms, and peak just prior to voluntary meals (Cummings et al., 2001). In rodents, peaks are evident just prior to the dark phase of the light cycle, largely coinciding with a typical feeding bout (Drazen, Vahl, D'Alessio, Seeley, & Woods, 2006). Such findings point to a strong relationship between ghrelin and the sensation of hunger.

It should be noted that the metabolic and non-metabolic effects of ghrelin deficiency can be compensated for if the deficiency is present from an early stage of development (Sun, Butte, Garcia, & Smith, 2003). However, rendering an animal ghrelin deficient in adulthood does not neutralize the effects of exogenously administered ghrelin. Deletion of ghrelin receptors caused a reduction in diet-induced obesity (Zigman et al., 2005) and similar effects on feeding were observed in animals treated with GHSR antagonists (Asakawa et al., 2003), or anti-ghrelin antibodies (Nakazato et al., 2001). These results suggest that early developmental insults to functional ghrelin or GHSRs can be compensated for by alternative neural mechanisms due to the redundancy of orexigenic networks (Depoortere, 2009).

Food reward

In many individuals, caloric intake is not in balance with metabolic rate and energy expenditure, resulting in either an anorexic or more likely, an obese phenotype. The alarmingly high prevalence of obesity suggests that additional factors may act in concert with homeostatic mechanisms to influence food intake. Abundant and readily available highly-palatable (i.e. appetizing) food sources that act upon central nervous system (CNS) reward circuitry may serve to entice individuals into unhealthy eating patterns, based on the hedonic properties of feeding rather than nutritional need. In an evolutionary context such complementary drives would be beneficial, as seeking out high calorie and pleasant tasting food would likely promote survival. Such a mechanism would also likely steer an animal away from poisonous foods. A dysregulation of this system with failure to adapt to the ever-increasing obesogenic environment poses some important health risks.

Palatable food, typically rich in sugar, fat, and/or salt, is particularly rewarding when consumed. These types of food offer a quick source of energy that is short-lasting, however, they also produce pleasure and gratification (Berridge & Robinson, 1998). Long-term consumption of such foods can lead to compulsive overeating, thereby contributing to the development of obesity and type II diabetes. Further, obese individuals exhibit a stronger preference for diets that are high in fat and carbohydrates relative to non-obese individuals (Drewnowski & Popkin, 1997).

Interestingly, overconsumption of highly palatable food leads to behavioural and physiological changes very similar to those seen in individuals addicted to drugs of abuse. Such analogous tendencies, including a lack of control and sensitization, suggest that the mesolimbic dopamine circuitry may be highly involved in the concept of "food addiction", for which scientific evidence is rapidly accumulating (Volkow & Wise, 2005). In fact, without

functionality of the brain's hedonic reward pathways, inherently tasty foods rapidly lose their appeal (Berridge, 2009).

Mesolimbic dopamine regulation of food reward

Experiencing pleasure or reward from eating is thought to be governed by the mesolimbic dopamine system. This primitive reward circuit in the brain consists of dopamine (DA) neurons that originate in the VTA and project predominantly to the nucleus accumbens (NAc). VTA dopamine neurons also contain afferents to multiple brain nuclei including the amygdala, prefrontal cortex (PFC), and hippocampus (Kelley & Berridge, 2002). Other natural and artificial rewards such as sex and drugs of abuse are thought to activate this same circuitry, resulting in reinforcement of motivated behaviours. The role of DA in eliciting and/or maintaining such behaviours has been thoroughly investigated in recent decades, but is yet to be fully understood.

As suggested by Berridge (2009), the 'liking' of a particular food is triggered by the action of endogenous opioid release in the NAc upon consumption. However, the subjective pleasure experienced must be translated into a 'wanting' or motivation to obtain that food in the future in order to have an influence on food intake, an effect largely dependent on the dopaminergic system. A recent study by Barbano et al. (2009) demonstrated such a dissociation by administering naloxone (an opioid antagonist) or flupenthixol (a DA D1/D2 receptor antagonist) to rats trained to traverse a straight alley runway or to perform instrumental behavior in an operant conditioning task to obtain food reward. Blockade of DA receptors appeared to affect the threshold of the cost/benefit ratio, such that rats were less likely to bar press for palatable food pellets. Blockade with naloxone on the other hand, resulted in a decreased consumption of palatable foods, not associated with the effort required to obtain it (Barbano, LeSaux & Cador, 2009).

Incentive salience can be defined as the ability of a reward or cues that predict reward (Schultz, 1998) to elicit motivated behaviours (Berridge & Robinson, 1998; Everitt & Robbins, 2005; Salamone & Correa, 2002). Once associated with 'liking' of a reward, these predictive cues can come to trigger cravings, or 'wanting'. Such cues can become so potently motivating that simply the thought of the sight, taste, and smell can trigger a craving in humans (Pelchat et al., 2004).

The neurochemical basis of such motivation appears to be the release of DA from the stimulation of neuronal cell bodies in the VTA, causing a downstream efflux of DA in the NAc. Salamone and colleagues propose that the amount of NAc DA is positively correlated with the physical effort an animal is willing to exert via an instrumental task to obtain a food reward (Salamone et al., 1991; Salamone & Correa, 2002). The same group demonstrated that DA depletion within the NAc reduced the motor effort exerted to obtain food reward and significantly decreased operant responding for food.

Unlike research conducted on drug addiction which demonstrated a central role for NAc DA in self-administration, early NAc depletion studies did not support a role for this region in free feeding paradigms using regular chow in rodents (Ikemoto & Panksepp, 1996). Interestingly, such animals still displayed robust responding for food in an operant paradigm (Balleine & Killcross, 1994). More recently, a strong case has again been made for the contribution of DA to food reward, although it is more widely accepted that the relationship between DA systems and reward are quite complex (Saper, Chou & Elmquist, 2002).

Actions of ghrelin on mesolimbic circuitry

Emerging evidence now supports the idea that peripherally synthesized energy regulatory peptides such as ghrelin play a role in the regulation of motivation to obtain food reward. For

example, administration of exogenous ghrelin increases food intake in animals that are satiated (Tschop et al., 2000; Nakazato et al., 2001), suggesting the possibility that that ghrelin is acting on areas other than homeostatic networks to enhance the salience or palatability of food sources. Further, early studies demonstrated that ghrelin selectively increased the consumption of foods high in caloric value, particularly high-fat foods. When rats were given acute ICV injections of ghrelin, and given a choice between two types of food, they chose fat more frequently (Shimbara et al., 2004). Considering this evidence, perhaps ghrelin acts on mesolimbic DA circuitry not to maintain energy balance, but to influence the motivation to select and consume highly palatable foods.

In support of this hypothesis, the presence of GHSR message has been reported in the ventral tegmental area (VTA), an area known to be important for reward and reinforcement (Guan et al., 1997; Zigman et al., 2006). Numerous studies have shown that ghrelin binds to the VTA, is colocalized with DA neurons, and when administered acutely it produces a robust feeding response (Naleid et al., 2005), similar to what would be seen following a sustained period without food (Abizaid et al., 2006). Conversely, blocking ghrelin using a selective GHSR antagonist, BIM28163, blunted peripherally induced ghrelin food intake, reflecting less of an interest in food (Abizaid et al., 2006).

Highlighting the interaction of ghrelin with the mesolimbic DA system, a recent study has shown that systemic administration of ghrelin increases extracellular DA release in the shell region of the nucleus accumbens, using *in vivo* microdialysis (Quarta et al., 2009). These results are in concordance with earlier studies showing that ghrelin administered directly to the VTA causes a rapid increase in excitatory inputs to VTA DAergic neurons (Abizaid et al., 2006) thereby increasing extracellular concentration of DA in the NAc (Jerlhag et al., 2006). Results of

the latter study also showed an increase in locomotor activity in ghrelin treated animals, an effect consistent with heightened DA transmission.

Caloric restriction (CR), which results in an increase in plasma ghrelin levels, has been shown to potentiate locomotor responses to abused drugs, such as cocaine and amphetamine (Carr et al., 2003). The mechanism by which CR causes such increases in locomotor responses to a drug challenge was explored in a series of studies conducted by Carr and colleagues (2003). ICV injections of DA agonists increased locomotor activity in CR rats more than in satiated rats, and FOS immunoreactivity, a marker of neuronal activation, was increased in the NAc after CR and agonist administration. Adaptation of the DA system in response to CR appears to be responsible for the increase in locomotor responses to drugs that act on mesolimbic circuitry, and it is possible that high levels of ghrelin present under such conditions contribute to this effect.

As mentioned previously, food intake and drug addiction share many common features (Volkow & Wise, 2005). Consumption of both highly palatable foods and drugs of abuse both result in DA overflow in mesolimbic circuits (DiChiara et al., 2004), and CR also increases the rate of responding for psychostimulant drugs (Carroll, France & Meisch, 1979; Carr, 2002). An interaction between ghrelin and dopamine circuitry may be responsible for such behavioural effects.

Conditioned place preference (CPP) is a classical conditioning paradigm that is used to evaluate the preference for an environment that has been paired with a particular reward. Rats that are pretreated with systemic ghrelin display an augmented place preference for an environment paired with a sub-threshold dose of cocaine, compared to rats that received saline pretreatment (David, Wellman & Clifford, 2007). In addition, pretreatment with ghrelin increased the hyper-locomotion associated with cocaine administration (Wellman, Hollas &

Elliott, 2008). Additional studies using alcohol reward demonstrated that central ghrelin administered to tegmental areas increased alcohol intake in a free choice paradigm, whereas ghrelin antagonists reduced responding for alcohol reward (Jerlhag et al., 2009).

Ghrelin has also been shown to have an effect on the hedonics of feeding in humans. When injected peripherally with physiologically relevant doses of ghrelin, feeding responses were increased in addition to self-reports of hunger and imagery of preferred foods (Wren at al., 2001; Cummings et al., 2004). Ghrelin also increases brain activity in areas within the mesolimbic dopamine system, such as the NAc and the amygdala (Malik et al., 2008). A recent genetic association study has revealed that certain single nucleotide polymorphisms on the GHSR-1 gene were associated with both heavy alcohol consumption and body mass (Landgren et al., 2008). Taken together, results from human studies implicate ghrelin in the enhancement of the palatability of food that is offered (Druce et al., 2006).

Evaluating the motivation to obtain food reward

One of the most common paradigms used to assess the motivation, or the amount of work an animal will conduct to obtain food reward, is the operant conditioning paradigm. Operant conditioning is characterized by a reinforcing stimulus (S) that is contingent upon a response (R). If a stimulus has reinforcing properties, it will increase the probability of the occurrence of a response. The motivation to obtain both natural and artificial rewards has been studied extensively using this paradigm in rodents. In an attempt to determine the efficacy of a reinforcer, the use of a progressive ratio (PR) schedule of responding has proven valuable (Hodos, 1961; Richardson & Roberts, 1996). In a PR schedule experiment, the response contingency required to obtain a reward is progressively increased after administration of

reinforcement. This paradigm requires that the animal increase the amount of effort required to obtain the same magnitude of reward, thereby increasing the cost/benefit ratio.

The efficacy of a given reinforcer (e.g. a food pellet) is typically defined in terms of the breakpoint (BP). The BP is the point in the series at which responding ceases (i.e. the final ratio completed by an animal) and reflects the maximum effort that the animal is willing to exert to obtain a particular reward (Richardson & Roberts, 1996). A stimulus that elicits a higher BP is considered to be more reinforcing than a stimulus that animals cease responding for earlier. Cessation of responding is related to both satiation and the increasing demands placed on the animal by the schedule of reinforcement. Indeed, some researchers use an exponential progressive ratio (PR) schedule to ensure that the BP will be achieved before the animal becomes satiated via reinforcements, particularly when using liquid reinforcements (Sclafani & Ackroff, 2003). Results from experiments utilizing a number of different reinforcers can be compared to examine their relative reinforcing values.

Rationale for Present Thesis

A substantial amount of evidence supports the role for ghrelin in the hypothalamic regulation of energy balance, with most of its regulatory functions being attributed to its action on orexigenic NPY and AgRP neurons in the ARC (Cowley et al. 2003; Guan et al., 2003; Kageyama et al., 2010). Support for the interaction of ghrelin with the mesolimbic DA system to influence the appetitive and motivational aspects of food intake has grown considerably in recent years, yet we are still far from understanding the full implications of such an interaction. Early studies indicate that central administration of ghrelin can induce plastic changes in areas of reward circuitry such as the VTA (Abizaid et al., 2006), increase dopamine release in the NAc (Jerlhag et al., 2007), and produce a robust feeding response in satiated rats (Naleid et al., 2005).

To date, there are few studies that examine the influence of ghrelin on the motivational aspects of feeding, especially with regards to highly palatable food, and likely a key variable in the development of obesity. A recent study has shown that acute systemic ghrelin administered prior to a PR test in mice increased the breakpoint in responding for a high-fat diet (HFD) in an operant paradigm, indicating an important role for ghrelin in instrumental behaviours leading to food reward (Perello et al., 2009). The current series of experiments was conducted to further explore the role of chronic central ghrelin administration on free feeding behaviours, macronutrient preference, and the motivation to obtain food reward as measured by operant behaviour.

Experiment 1

The main goal of this experiment was to determine the effects of intra-VTA ghrelin on food intake and body weight under standard *ad libitum* conditions. Male rats received either saline, low ghrelin (3nM/day), or high ghrelin (10nM/day) directed at the VTA via intracranial cannulae connected to a 14 day osmotic mini-pump. Intake of regular chow and body weight gain were measured over the two week infusion period. Since acute ghrelin administered into this region induces a robust feeding response (Naleid et al., 2005), it was hypothesized that chronic ghrelin delivery would also increase food intake and resulting increase in body weight.

Experiment 2

A second study was conducted to determine the effects of chronic intra-VTA ghrelin on macronutrient diet preference. Male rats were implanted with guide cannulae directed at the VTA connected to a 14-day mini-pump delivering either saline, ghrelin (10nM/day; most effective dose in the previous experiment), or a ghrelin receptor antagonist, [D-Lys3]-GHRP-6 (200nM/day). The rats were given access to three isocaloric diets simultaneously available *ad*

libitum in the home cage (see Appendix A for nutritional composition of diets). Daily caloric intake of each diet and body weights were recorded throughout the testing period and for 1 week following treatment. Given that ghrelin has been shown to preferentially increase the consumption of a high-fat diet (Shimbara et al., 2004), we hypothesized that ghrelin treated rats would choose high-fat as their primary source of calories, as well as substantially increase their body weight, compared to saline controls. Conversely, administration of a ghrelin antagonist was hypothesized to blunt the preference for the high-fat diet and in turn cause a reduction in body weight gain.

Experiment 3

A third objective was to examine the effects of ghrelin on the motivation to obtain food reward using an operant conditioning paradigm. Male rats were again implanted with VTA cannulae attached to 14-day mini pumps and received either saline or ghrelin (10nM). Prior to surgery, rats were trained to bar press on a fixed-ratio 1 (FR1) schedule of responding. During the infusion period, rats were kept on an *ad libitum* feeding schedule, and tested again on an FR1 to compare rates of pressing before and after ghrelin administration. It was hypothesized that ghrelin treated rats would increase their rate of bar pressing for chocolate-flavoured pellets following treatment (despite being satiated), whereas, saline animals would exhibit similar or reduced rates of bar pressing during the testing phase.

Experiment 4

To further examine the influence of ghrelin on the motivation to obtain a highly palatable food source, we measured how much work rats were willing to perform to obtain chocolate-flavoured pellets in a progressive ratio operant paradigm. Male rats were trained in operant chambers prior to surgery and were subsequently implanted with cannulae as described in

experiment 3, but also included a third group treated with [D-Lys3]-GHRP-6 (200nM/day). Using a PR under which the response required to obtain a food reward increased exponentially after the receipt of each reward, we examined how much effort the rats were willing to exert to obtain the food pellets. We hypothesized that ghrelin treated rats would display an increased motivation to obtain chocolate pellets, as determined by an increase in breakpoints during treatment. Alternatively, the [D-Lys3]-GHRP-6 treated rats would reduce their level of responding following treatment, suggesting an important role for ghrelin in the motivation to obtain palatable foods.

Materials and Methods

Animals

Male Long-Evans rats (~90 days old; $N = 70$) obtained from Charles River Laboratories (St. Constant, Quebec, Canada) were used for all experiments. Rats were individually housed in transparent Plexiglass cages (48cm X 26cm X 20cm) in a temperature and humidity controlled environment (22°C and 45-55%, respectively). Rats were left undisturbed for one week to allow for acclimatization to vivarium conditions. All rats were maintained on a standard 12 hour light dark cycle (lights on at 08:00) with *ad libitum* access to standard laboratory rat chow and water supply, except when indicated. All experimental procedures complied with the Canadian Council on Animal Care (CCAC) guidelines and were approved by the Local Ethics Committee at Carleton University.

Surgical Procedures

Male rats were anaesthetized with isofluorane and oxygen (4:2 for induction; 2:2 for maintenance) for the duration of the procedure. The rats' head and a small dorsal region was shaved and the rat was mounted into a small animal stereotaxic apparatus (Kopf Instruments,

Tujunga, CA) positioned atop a heating pad. The scalp was cleaned with both Surgiprep and Priviodine to provide an aseptic canvas; tear gel was also applied to prevent dehydration of the eyes. A midline incision was made using a 10mm scalpel and the skin and periosteum were retracted to enable clear visualization of Bregma. Guide cannulae were implanted using coordinates obtained from a standard atlas of the rat brain (Paxinos & Watson, 1998).

A twenty-six gauge stainless steel unilateral guide cannulae (Plastics One, Roanoke, VA) attached via polyethylene catheter to an osmotic mini-pump (Alzet Mini-Osmotic Pump Model 2002; DURECT; flow rate, 0.5ul/hr for 14 days) was implanted into the ventral tegmental area (coordinates: AP -5.3mm, ML + 2.0mm, DV -7.6mm). Mini-pumps were filled with 0.2 ml of either sterile saline (0.9% NaCl), ghrelin solution (Peptides International; 10nM/rat/day), or a ghrelin receptor antagonist ([D-Lys3]-GHRP-6) solution (200nM/rat/day). Three holes were drilled for implantation of jeweller screws, serving to anchor the cannula to the surface of the skull. The cannula and the screws were affixed to the skull using dental cement.

Once the cement was dried completely, the dorsal portion of the skin was separated from the muscle using blunt dissection in order to implant the mini-pump subcutaneously. Following closure of the incision with surgical sutures, Polysporin and Lidocaine were applied to the surgical site to prevent bacterial infection and pain, respectively. Rats were also injected with Metacam (0.1 ml) to provide post-operative analgesia. Upon completion of surgery, rats were placed in clean cages with cob bedding atop a heating pad and maintained in a recovery area until they awoke. Rats perceived to be in good health were returned to the vivarium and monitored closely to ensure optimal recovery.

Operant Training and Testing

Prior to surgery, animals were food restricted to 90% of their initial body weight and trained to bar press on a fixed-ratio 1 (FR1) schedule of responding in standard operant conditioning chambers (Colbourne Instruments) equipped with one nose poke portal flanked by two retractable levers. Every correct press of the active lever resulted in the delivery of one 45 mg highly-palatable chocolate flavoured food pellet (Bioserv; see Appendix A). Each training session was 30 minutes in duration and was conducted between 0800 and 1200 hr. Once each rat reached a stable level of responding on an FR1 schedule during training (~12 days), stereotaxic surgery was conducted. Following recovery from surgery, rats were tested again in the operant chambers to determine the effects of chronic ghrelin infusion on operant responding.

In experiment 4, once each rat reached a stable level of responding on an FR1 schedule during training (~12 days), they were moved to a fixed ratio 2 schedule and again up to a fixed ratio 4 schedule. Finally, a progressive ratio (PR) probe session was conducted to establish a break point (BP). During the infusion period, these rats were tested once on both an FR1 and FR4 schedule, and 3 times on the PR ratio schedule to examine the amount of effort each rat was willing to exert to obtain one highly-palatable food pellet.

Experiment 1

Experiment 1 assessed the ability of chronic ghrelin administration into the VTA to induce an increase in food intake and body weight. Twenty-four adult male rats (250-280 g) were single housed and received *ad libitum* chow and tap water. Following a 7 day baseline period measuring food intake and body weight, each rat received an indwelling unilateral guide cannula directed at the VTA. Each cannula was connected to an osmotic mini-pump delivering one of three doses of ghrelin (0, 3nM, 10nM). The rate of delivery was 0.5ul/hour/day for a total of 14

days. Food intake and body weight was monitored every 24 hours throughout the infusion period.

Experiment 2

Experiment 2 examined the effects of chronic intra-VTA ghrelin and a ghrelin receptor antagonist on macronutrient preferences in rats. Twenty-six adult male rats (250-280 g) were single housed and exposed to three different (isocaloric) diets simultaneously present in the home cage: 1) a 70% carbohydrate diet, 2) a 60% fat diet, and 3) a 60% protein diet (Harlan-Teklad Diets; see Appendix B). After baseline measurement of diet intake and body weight, animals were randomly assigned to 1 of 3 treatment groups and were implanted with 14-day osmotic mini-pumps delivering either 10 nM/day of ghrelin (Peptides International; N=9), or 200 nM/day of ghrelin antagonist ([D-Lys3]-GHRP-6; Geneway; N=9), or sterile saline (NaCl; N=8) into the VTA. Following surgery, daily intake of each macronutrient and body weight gain for the 14 days of treatment and 6 days post-infusion were recorded.

Experiment 3

Experiment 3 was designed to assess the effects of chronic intra-VTA ghrelin on the motivation to obtain highly palatable food in a traditional operant conditioning paradigm. Twenty adult male rats (250-280 g) were single-housed and received *ad libitum* chow and water during the acclimatization week, during which baseline food intake and body weight were recorded. Rats were subsequently calorically restricted to ~50% of their daily food intake until they reached 90% of their body weight. Once this target weight was reached, training in the operant chamber commenced. Daily 30 minute training sessions were conducted until animals reached a stable level of responding on a fixed ratio 1 schedule of reinforcement, at which point rats received stereotaxic surgery, as outlined in experiment 1. Mini-pumps were filled with either

saline (N=10) or ghrelin (N=10, 10nM/day), the dose found to be effective in experiment 1. Following surgical procedures, all animals were returned to an *ad libitum* feeding schedule and food intake and body weight were recorded daily. On days 4, 5 and 6 post-surgery, rats were tested in the operant chambers on an FR 1 schedule to allow comparison to baseline levels of responding.

Experiment 4

Experiment 4 examined the effects of ghrelin in the VTA on the motivation to obtain a highly palatable food reward in a PR operant paradigm. Twenty four adult male rats underwent caloric restriction and operant training procedures similar to those outlined in the previous experiment, however, animals were tested for one session on a PR schedule of responding prior to surgery. During surgery, rats received mini-pumps filled with either saline (N=8), ghrelin (10nM/day; N=8), or [D-Lys3]-GHRP-6 (N=8). Once rats recovered from surgery, they were returned to calorie restriction to examine the effects of chronic VTA ghrelin and food restriction on operant responding for food reward on a PR schedule. On day 4 post-surgery, rats were tested on a FR 1 schedule to allow for comparison to baseline measures, and on day 6 rats were switched to an FR 4 schedule. Finally, on days 8, 11, and 14 post-surgery, rats were tested on a PR schedule of responding, during which the requirement to obtain one food pellet increased exponentially following every correct response according to the series: 1, 2, 4, 6, 9, 11, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219 etc., derived from the following equation: $[5e^{(\text{injection number} \times 0.2)}]-5$. Rats remained in the operant chambers until 1 hour had passed without an instrumental response. BP was defined as the last ratio that the rat successfully completed to receive a reinforcer.

Histology

On completion of behavioural observations, rats were overdosed with Nembutal and transcardially perfused with 4 % paraformaldehyde (PFA). Extracted brains were post-fixed for 24 hrs in 4% PFA, followed by cryo-protection in a 30% sucrose solution in 0.1 M phosphate buffer solution and storage in 4°C until sectioning. To verify cannula placements, brains were frozen and sliced into 50uM coronal sections using a cryostat (LEICA CM1900). Coronal sections were mounted onto gel-coated slides and stained using cresyl violet.

Cresyl Violet staining. Each slide was submerged in the following series of solutions for 2 mintues each: 100% ethanol, 95% ethanol, and 70% ethanol. Sections were then rinsed in distilled water to remove excess ethanol and subsequently placed in a 1% cresyl violet solution. Sections were then rinsed in distilled water to remove excess cresyl and immersed in 0.8% acetic acid to allow for differentiation (~2-5 minutes). Sections were then placed in 70%, 95% and 100% ethanol for 2 minutes each, respectively. Upon completion of staining, slides were immersed in Clearene solution for 15 minutes prior to cover-slipping with clarion mounting media. Microscopic examination was then used to determine location of infusion sites.

Statistical analysis

All data are expressed as mean +/- SEM. Food intake, body weight, and operant responding data for experiments 1, 2, and 4 were analyzed using analysis of variance (ANOVA) tests, followed by post-hoc comparisons where significant differences were found. Since there were only two treatment groups in experiment 3, t-tests were used to analyze the data in that study. In all statistical analyses, a p-value of 0.05 or less was considered significant. For each experiment, rats with misplaced cannulae were excluded from statistical analysis (see Appendix C).

Results

Experiment 1: The effects of chronic ghrelin on ad libitum food intake and body weight

To investigate the effects of chronic ghrelin administration directly into the VTA on *ad libitum* food intake and body weight, rats received 14 days of continuous ghrelin infusion (low dose, 3nM; high dose, 10nM; or saline) into the VTA via osmotic mini pump. One-way ANOVA revealed that by the end of the baseline period, all rats were consuming a similar amount of chow daily ($F_{(2,18)} = 1.452$, $p = .260$, see Figure 1a) and displayed no significant differences in body weight ($F_{(2,18)} = .586$, $p = .567$, Figure 1b).

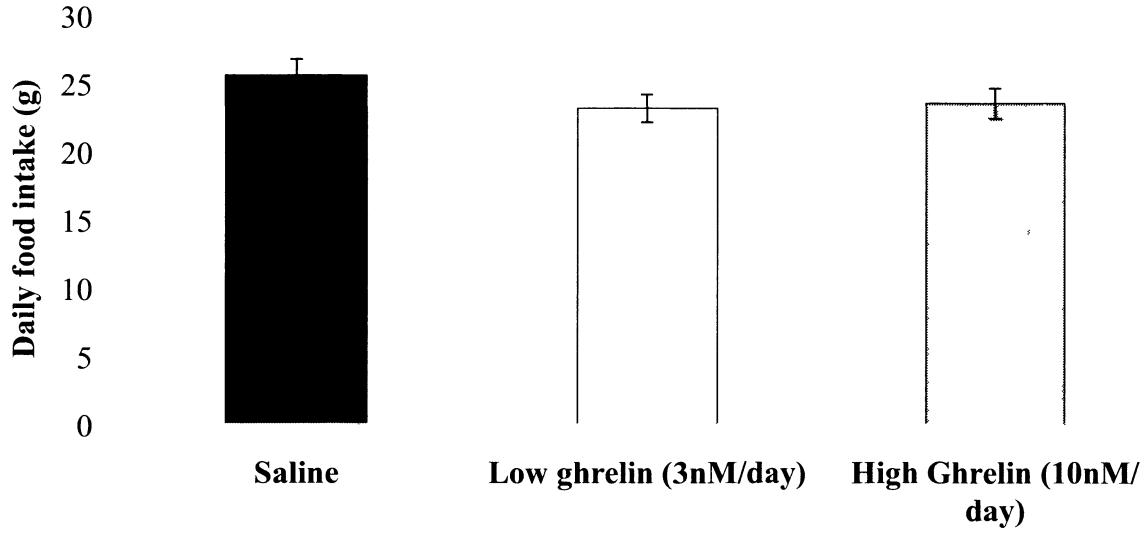


Figure 1a. Mean (+/- SEM) daily food intake during the baseline period prior to surgery. No differences were observed between treatment groups (Saline: $n = 8$; 3 nM ghrelin: $n = 8$; 10 nM ghrelin: $n = 8$).

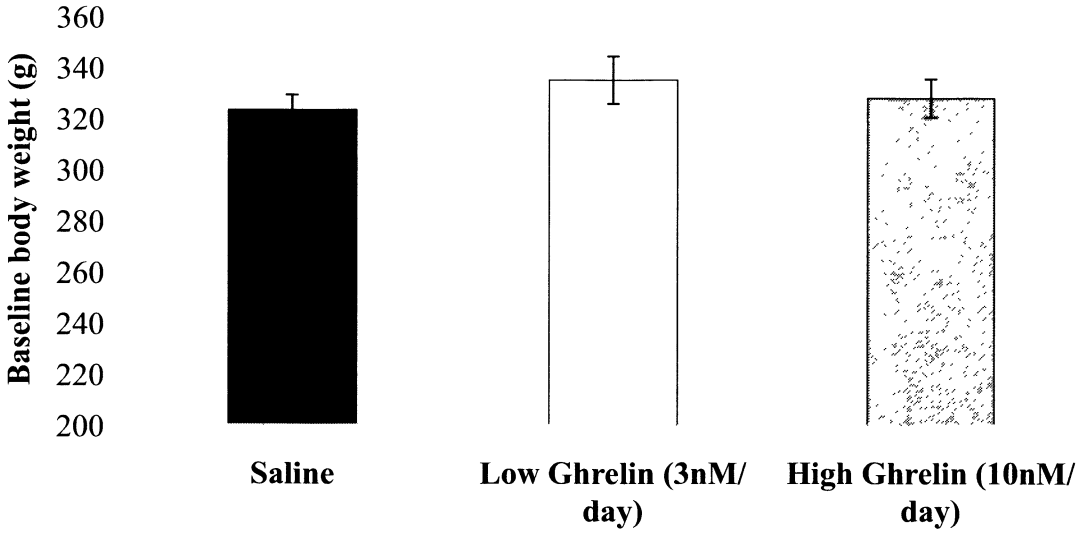


Figure 1b. Mean (+/- SEM) body weight during the baseline period. No differences were observed between treatment groups (Saline: $n = 7$; 3 nM ghrelin: $n = 8$; 10 nM ghrelin: $n = 6$).

A 3 (group) x 14 (day of treatment) mixed-design repeated measures ANOVA revealed a main effect of treatment group ($F_{(2,18)}$=4.337, p = .029) for change from baseline chow intake during the infusion period (Figure 2) . Fischer's LSD post-hoc analyses revealed that rats receiving a 10 nM dose of ghrelin consumed on average 6.2 g more chow per day across the treatment period than saline treated rats, who consumed 2.92 g more chow per day, relative to baseline intake (p = .009). The group infused with 3 nM ghrelin ate an average of 4.27 g more food relative to their baseline intake, but did not differ statistically from the 10 nM (p = .090) or the saline groups (p = .214).

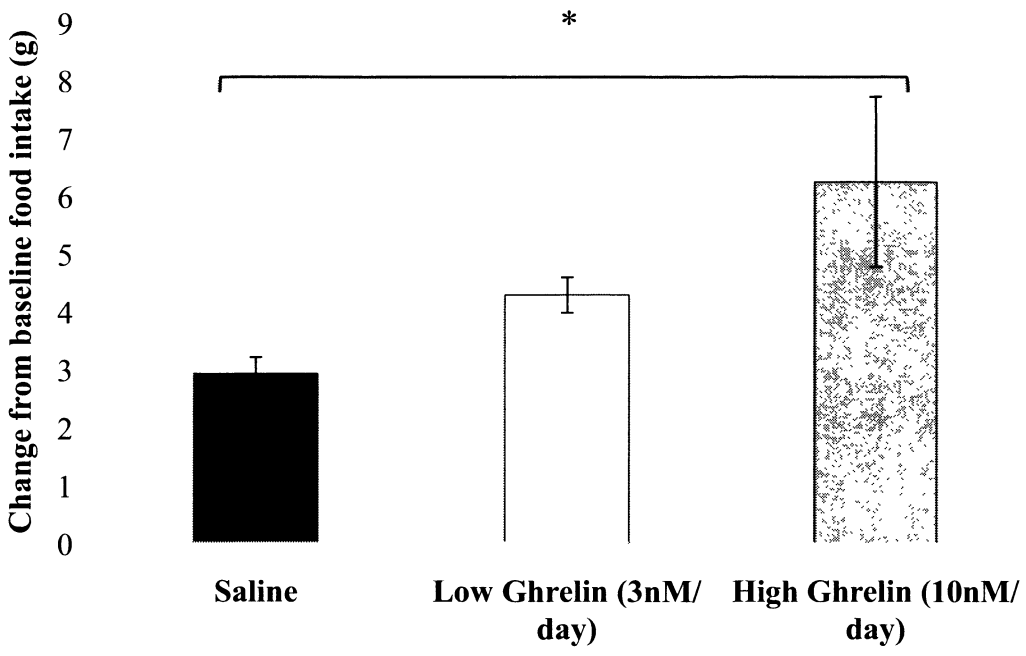


Figure 2. Mean (+/- SEM) change from baseline food intake during the infusion period. (Saline: $n = 7$; 3 nM ghrelin: $n = 8$; 10 nM ghrelin: $n = 6$). * $p < .05$.

A 3 x 14 mixed design repeated measures ANOVA demonstrated a main effect of treatment group ($F_{(2,18)} = 3.738$, $p = .044$) for body weight gain throughout the infusion period with no main effect of day ($F_{(2,18)} = 3.738$, $p = .061$) and no interaction ($F_{(2,18)} = .702$, $p = .553$). Fischer's LSD post-hoc analysis demonstrated that rats treated with 10 nM ghrelin gained more body weight per day during treatment (M= 7.17 g), compared to saline treated rats ($p = .017$; M = 5.9 g), and low ghrelin treated rats showed a marginally significant weight gain compared to saline treated rats ($p = .051$; M = 6.19), as depicted in Figure 3.

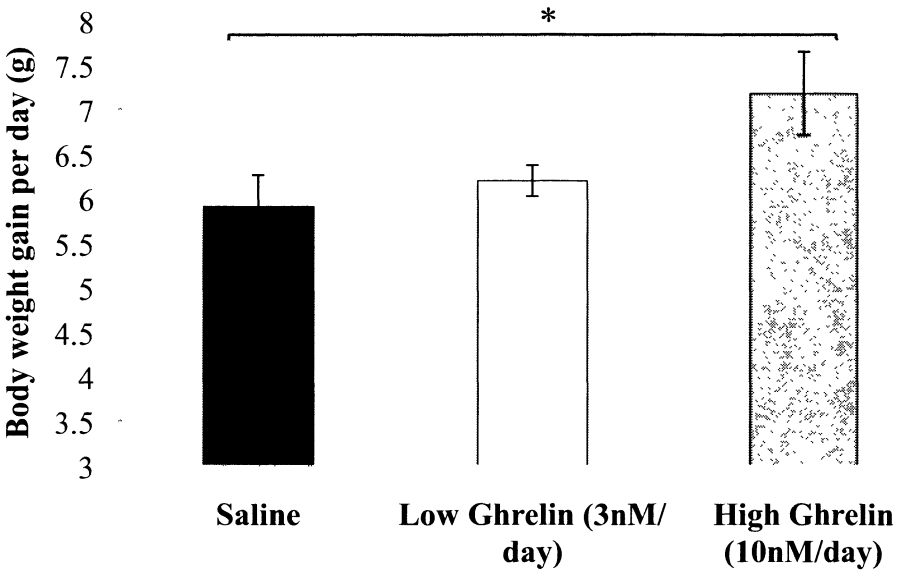


Figure 3. Mean (+/- SEM) body weight gain per day during the infusion period. (Saline: $n = 7$; 3 nM ghrelin: $n = 8$; 10 nM ghrelin: $n = 6$). * $p < .05$.

When total weight gain over the infusion period was examined by one-way ANOVA, high ghrelin treated rats exhibited the greatest total weight gain ($F_{(2,18)}$ =3.743, p = .021; M = 99.33 g), significantly different from the saline group (M = 81.71 g), but not different from the low ghrelin group (p = .179; M = 86.75 g), as shown in Figure 4.

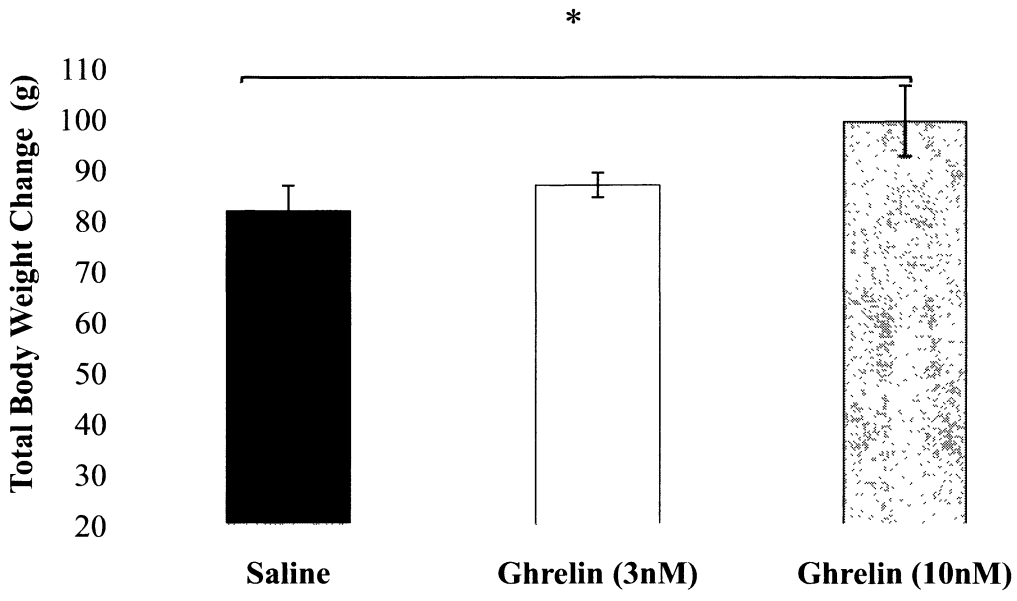


Figure 4. Mean (+/- SEM) total weight gain across the infusion period. (Saline: $n = 7$; 3 nM ghrelin: $n = 8$; 10 nM ghrelin: $n = 6$). * $p < .05$.

Experiment 2: The effects of chronic ghrelin on macronutrient diet preference

The effects of intra-VTA ghrelin or a ghrelin antagonist, [D-Lys3]-GHRP-6, on body weight gain and the composition of *ad libitum* caloric intake chosen from three isocaloric diets high in either fat, protein or carbohydrate were examined in this experiment. As depicted in Figure 5, body weight did not differ between groups by the end of the baseline period ($F_{(2,19)} = .273$, $p = .764$).

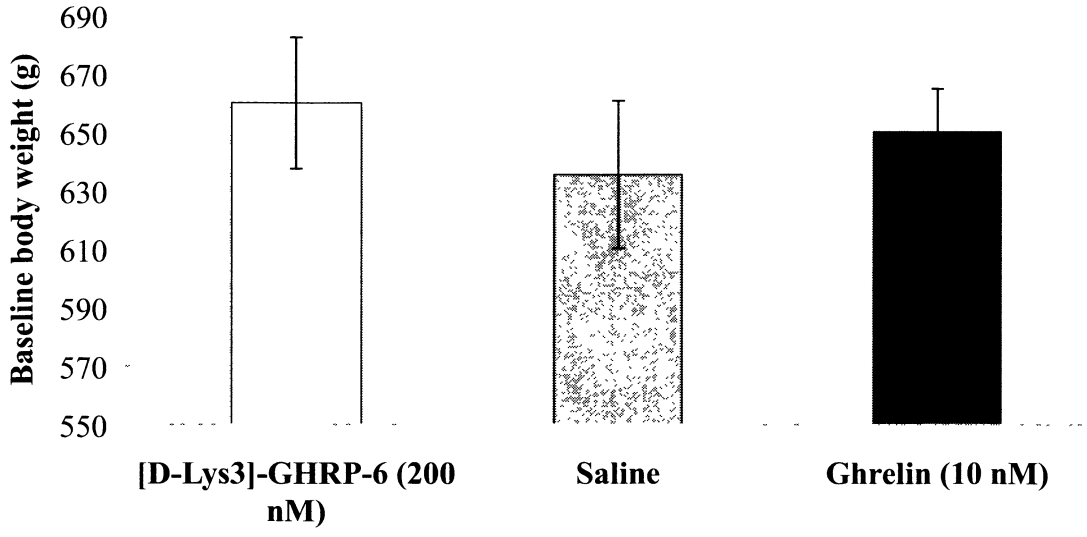


Figure 5. Mean (+/- SEM) body weight by the end of the baseline period. ([D-Lys3]-GHRP-6: n = 8; saline: n = 8; ghrelin: n = 8). * $p < .05$.

Differences between groups were found with regard to average body weight gain throughout the infusion period ($F_{(2,19)} = .9.947$, $p = .001$), as depicted in Figure 6. Fischer's LSD post-hoc analysis demonstrated that rats who received [D-Lys3]-GHRP-6 exhibited a decrease in body weight relative to both saline ($p = .0003$) and ghrelin treated rats ($p = .001$), whereas saline and ghrelin treated rats both gained weight, but did not differ significantly from each other ($p = .607$).

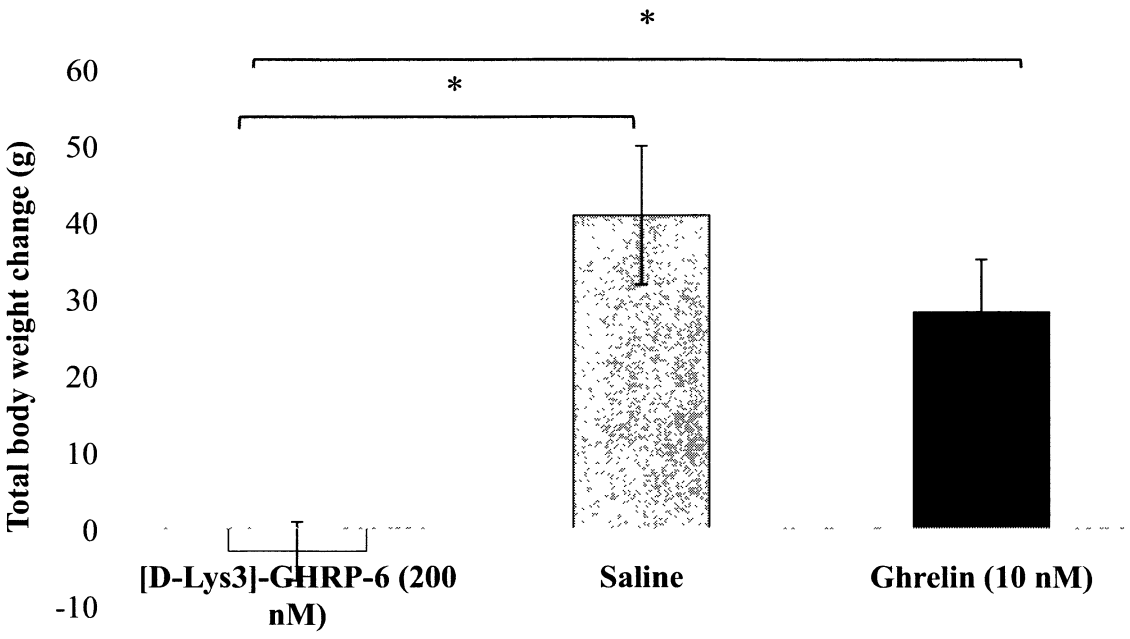


Figure 6. Mean (+/- SEM) body weight gain over the two-week infusion period. ([D-Lys3]-GHRP-6: $n = 8$; saline: $n = 6$; ghrelin: $n = 8$). * $p < .05$.

With regard to diet composition during the baseline period, groups did not differ in consumption of fat, protein, or carbohydrates ($F_{(2,19)} = .809$, $p = .5270$; Figure 7).

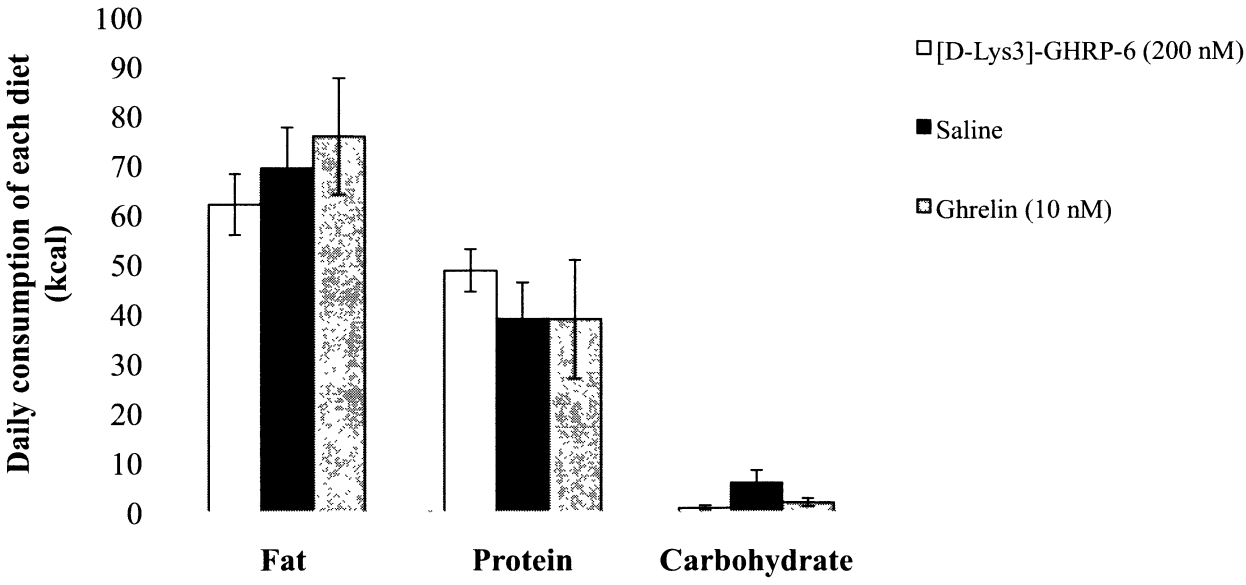


Figure 7. Mean (+/- SEM) kilocalories consumed from each diet per day throughout the baseline period. ([D-Lys3]-GHRP-6: $n = 8$; saline: $n = 6$; ghrelin: $n = 8$). * $p < .05$.

In order to examine the change in composition of daily caloric intake derived from each of the three diets across time, a mixed design repeated measures ANOVA (time x group x diet) was conducted across the baseline, treatment and post-treatment periods. Analysis revealed a significant group by time by diet interaction ($F_{(2,19)} = 172.780$, $p < .001$). Post-hoc analyses revealed that saline and ghrelin treated rats increased their daily caloric intake of the high-fat diet during the treatment period compared to the [D-Lys3]-GHRP-6 rats who showed a slight reduction in high-fat intake ($p = .0034$ and $p = .0029$, respectively; see Figure 8). Saline and ghrelin groups were not significantly different from each other ($p = .7533$).

During the post-treatment period [D-Lys3]-GHRP-6 treated rats did not show reduced high-fat intake relative to either the ghrelin group ($p = .5982$) or the saline group ($p = .9058$), and ghrelin treated rats ate a similar amount of high-fat diet as saline treated rats ($p = .6761$), as displayed in Figure 8.

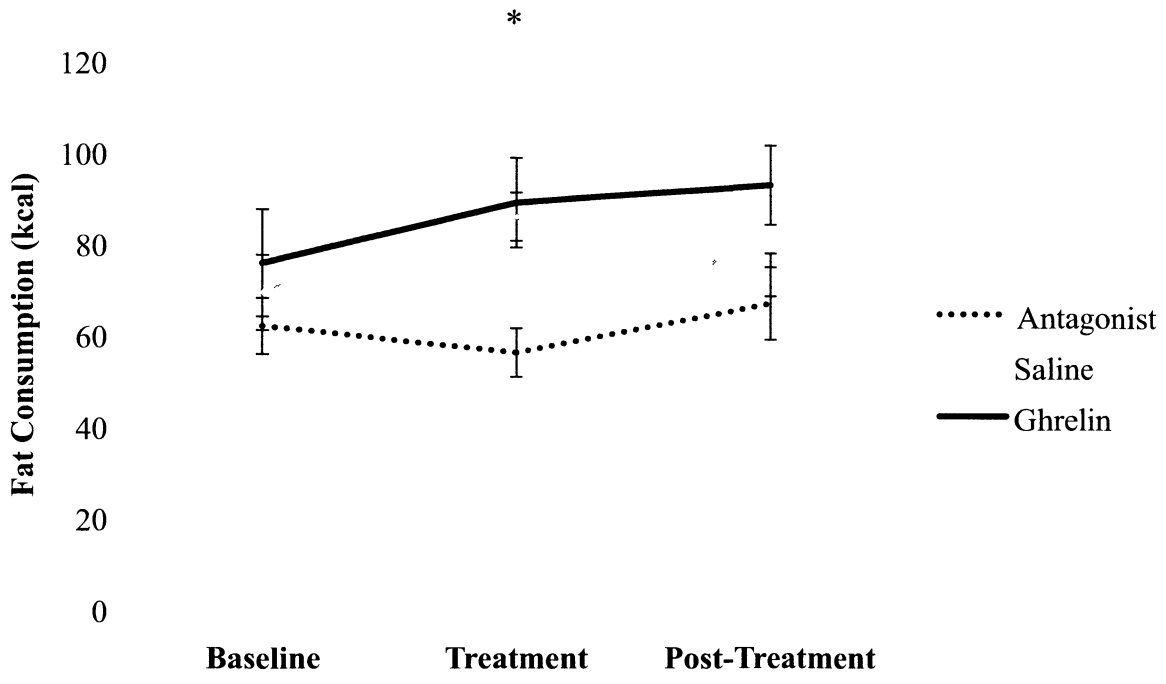


Figure 8. Mean (+/- SEM) kilocalories of fat consumed per day across baseline, treatment and post-treatment periods. ([D-Lys3]-GHRP-6: $n = 8$; saline: $n = 6$; ghrelin: $n = 8$). * $p < .05$.

As shown in Figure 9, protein consumption did not differ between groups during any of the time points examined. During treatment, [D-Lys3]-GHRP-6 treated rats ate similar amounts of high-protein diet per day compared to both the saline ($p = .1995$) and the ghrelin treated rats ($p = .0973$). Ghrelin and saline treated rats also consumed similar amounts of high-protein diet during the treatment period ($p = .6137$).

During the post-treatment period, no differences were found for protein consumption between the antagonist and the saline group ($p = .4968$) or the ghrelin group ($p = .1646$). Ghrelin treated rats and saline treated rats did not differ on high-protein diet consumption during post-treatment ($p = .4312$).

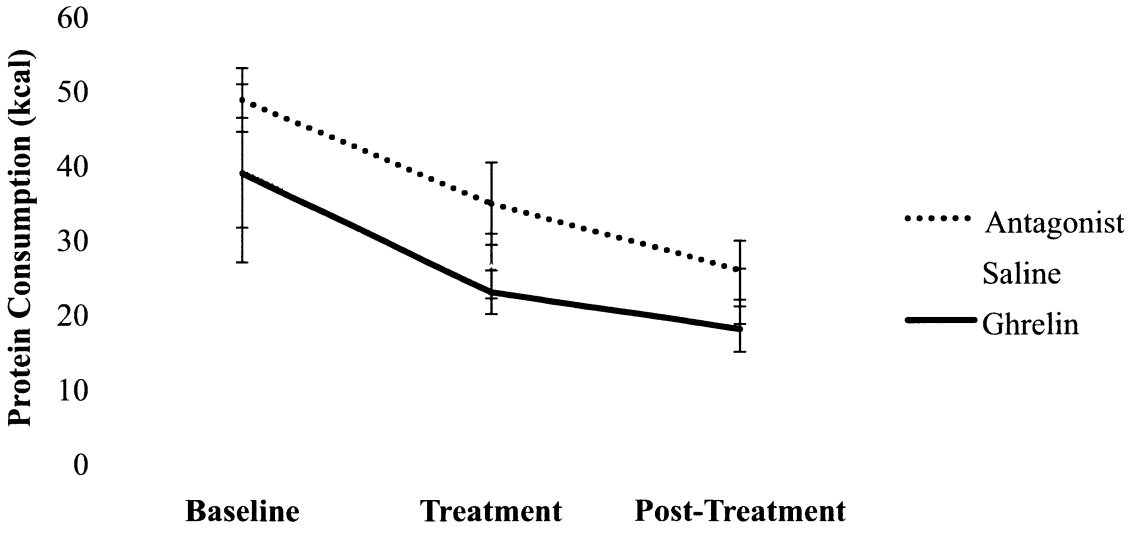


Figure 9. Mean (+/- SEM) kilocalories of protein consumed per day across baseline, treatment and post-treatment periods. ([D-Lys3]-GHRP-6: $n = 8$; saline: $n = 6$; ghrelin: $n = 8$). * $p < .05$.

During the treatment period, the saline group derived a greater number of calories per day from the high-carbohydrate diet, relative to both the antagonist group ($p = .0088$) and the ghrelin group ($p = .0166$), as depicted in Figure 10. During the post-treatment period, significant differences between groups were no longer present, and each group of rats consumed similar amounts of high-carbohydrate diet daily.

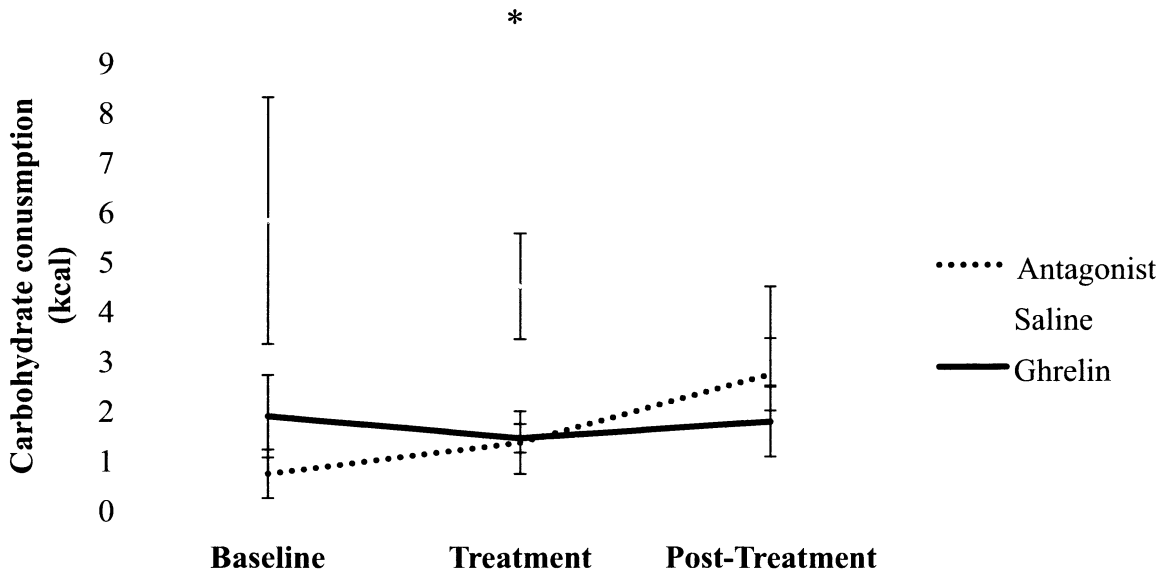


Figure 10. Mean (+/- SEM) kilocalories of carbohydrates consumed per day across baseline, treatment and post-treatment periods. ([D-Lys3]-GHRP-6: $n = 8$; saline: $n = 6$; ghrelin: $n = 8$). * $p < .05$.

Experiment 3: Effects of chronic ghrelin on body weight, food intake, and fixed-ratio responding

For experiment 3, t-tests were performed on both baseline body weight and total weight gain during the treatment period. Figure 11 illustrates that animals did not exhibit differing baseline body weights ($t_{(1,15)}$=.281, p = .783).

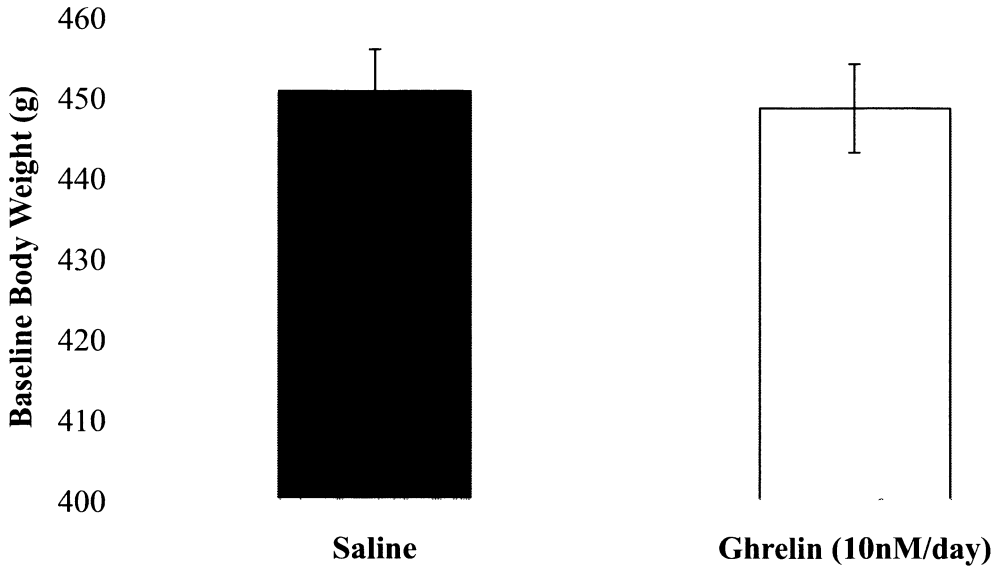


Figure 11. Mean (+/- SEM) baseline body weight. No differences were found between treatment groups. (Saline: $n = 7$; Ghrelin: $n = 10$).

However, by the end of infusion period, ghrelin treated rats gained a significantly greater amount of weight relative to the saline treated rats ($t_{(1,15)}$=-2.195, p = .044), as depicted in Figure 12.

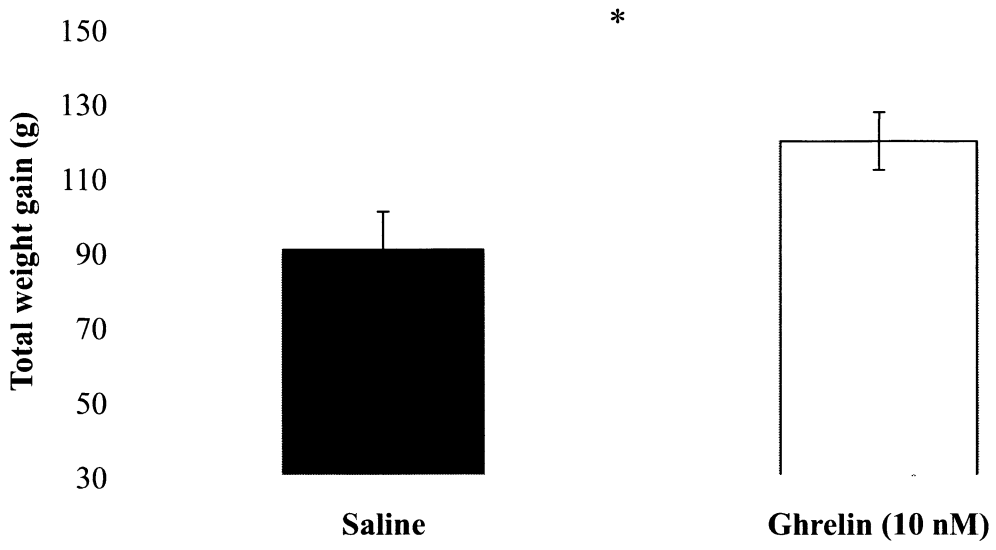


Figure 12. Mean (+/- SEM) total weight gain across the infusion period. (Saline: $n = 7$; Ghrelin: $n = 10$). * $p < .05$.

A repeated measures ANOVA revealed that ghrelin treated rats consumed more chow daily throughout the treatment period compared to saline treated rats ($F_{(1,15)}$=7.883, p = .013; Figure 13), consistent with greater total body weight gain over the two week period (Figure 12).

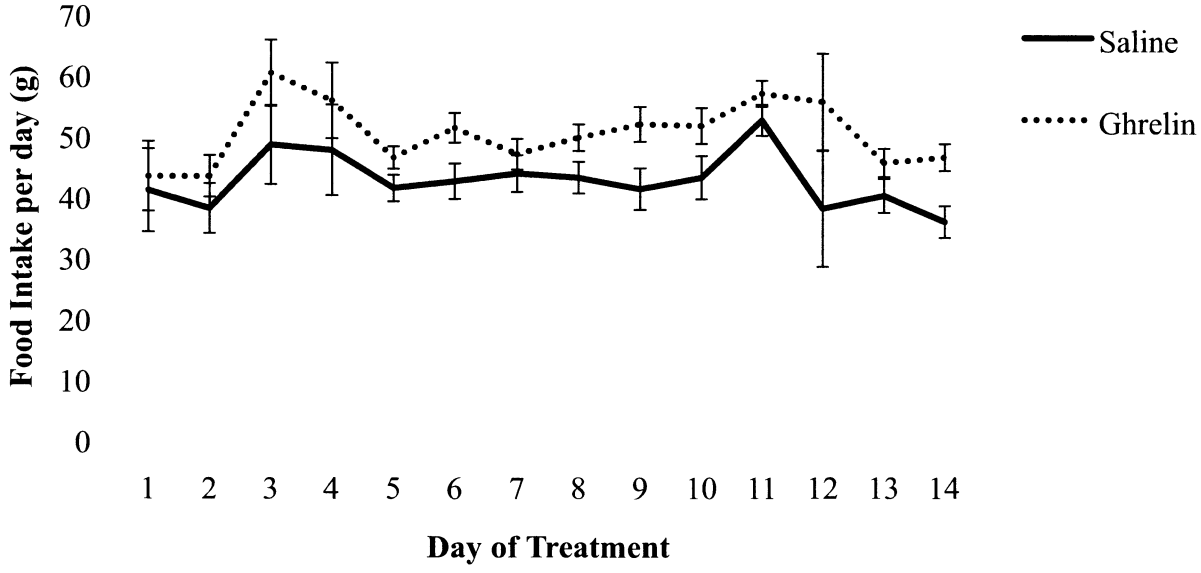


Figure 13. Mean (+/- SEM) daily food intake across the infusion period. (Saline: n = 7; Ghrelin: n = 10). * p < .05.

To explore the effects of ghrelin treatment on the percentage of baseline operant responding rats exhibited for a highly-palatable food source (i.e. chocolate flavoured pellets), a t-test was conducted. Results showed that ghrelin treated rats, under conditions during which they would be typically be satiated, showed a 30 percent increase in level of responding during ghrelin infusion ($t_{(1,15)}$= -3.048, p = .009), compared to their baseline rates. Alternatively, saline treated rats reduced their rate of responding on an FR1 schedule when food was made available *ad libitum* in the homecage (see Figure 14).

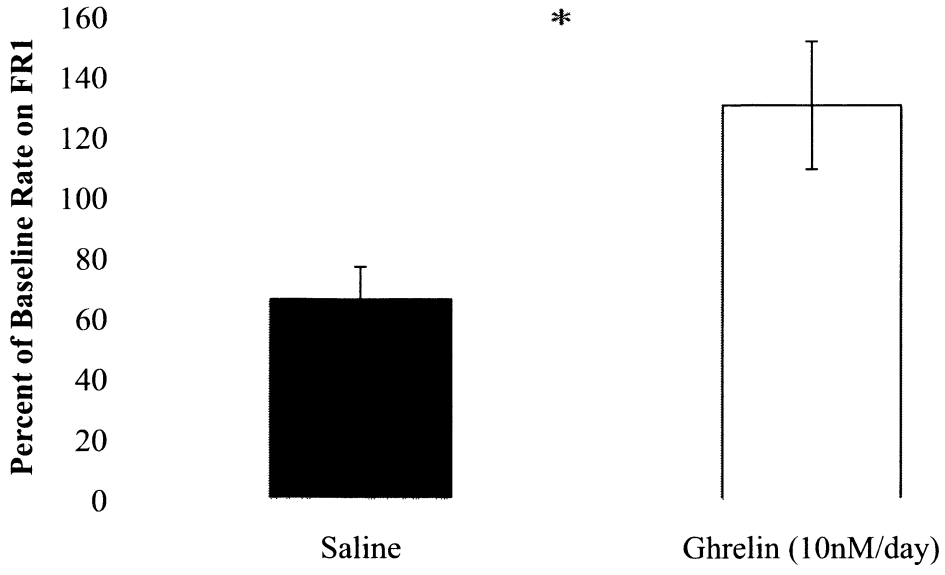


Figure 14. Mean (+/- SEM) percentage of baseline responding on an FR1 schedule of reinforcement. (Saline: $n = 7$; Ghrelin: $n = 10$). * $p < .05$.

Experiment 4: Effects of chronic ghrelin on motivation to obtain food reward

To follow the results of Experiment 3, we further explored the effects of ghrelin on the motivation to obtain highly-palatable food using a progressive ratio paradigm. According to one way ANOVA, all rats had similar body weights before any experimental manipulations were conducted ($F_{(2,19)}$=.812, p = .459), as shown in Figure 15.

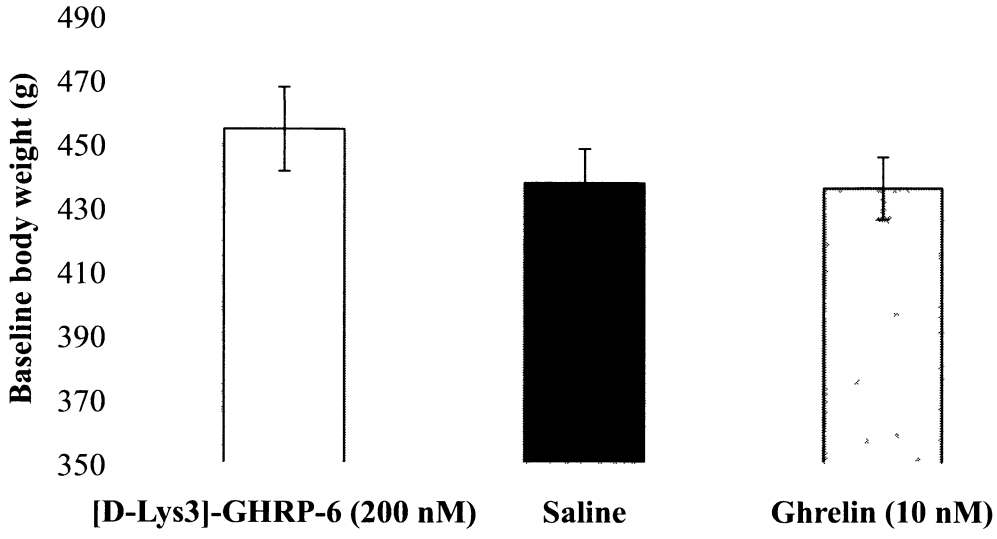


Figure 15. Mean (+/- SEM) baseline body weight. ([D-Lys3]-GHRP-6: $n = 7$; saline: $n = 8$; ghrelin: $n = 7$). * $p < .05$.

Progressive ratio testing was conducted on days 8, 11 and 14 of the infusion period. When cumulative bar presses were examined by two-way ANOVA repeated measures (time interval x group) within each session, no main effects of treatment group on day 8 ($F_{(2,19)} = .309$, $p = .738$; Figure 16) or day 11 ($F_{(2,19)} = 1.764$, $p = .196$; Figure 17) were found. There was a main effect of time interval on both days, as cumulative bar presses increase across the testing session (Day 8: $F_{(2,19)} = 35.930$, $p < .001$; Day 11: $F_{(2,19)} = 27.508$, $p < .001$). No interaction effects were found on days 8 or 11.

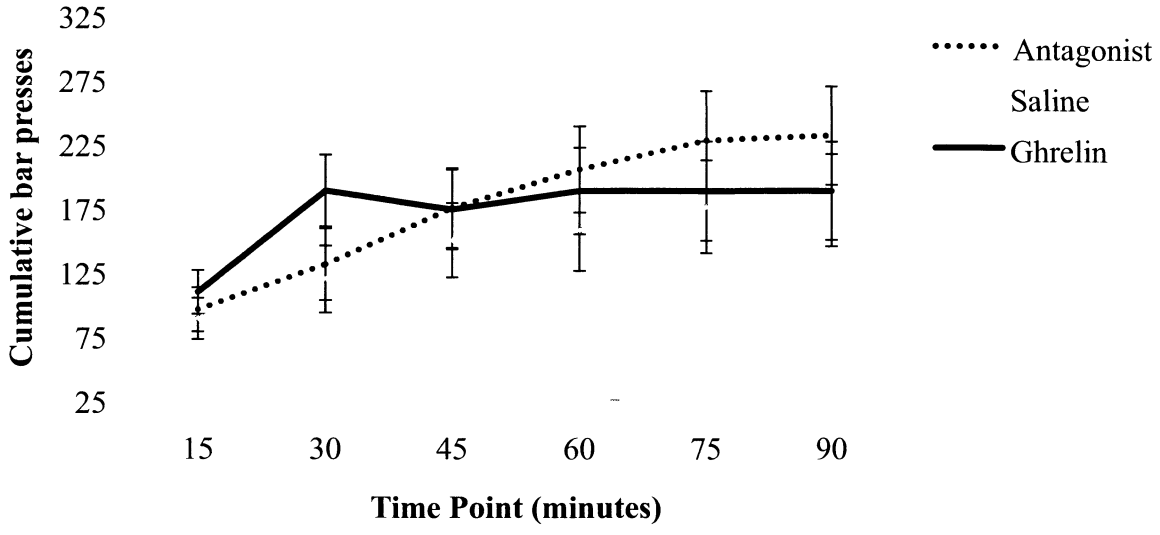


Figure 16. Mean (+/- SEM) cumulative bar presses on a PR schedule on first day of PR testing during infusion (Day 8). ([D-Lys3]-GHRP-6: $n = 7$; saline: $n = 8$; ghrelin: $n = 7$). * $p < .05$.

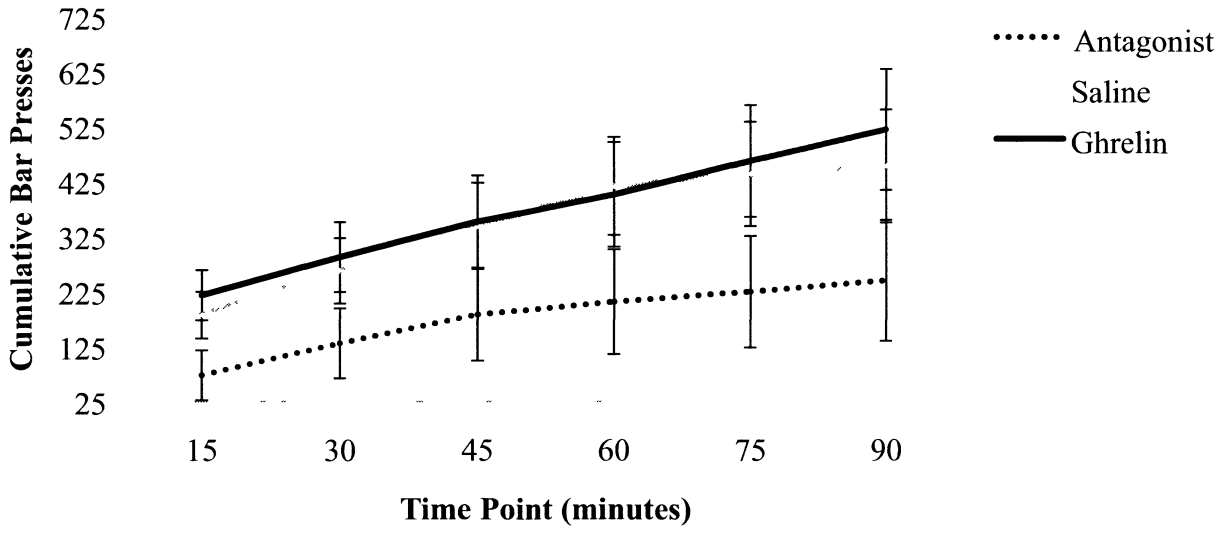


Figure 17. Mean (+/- SEM) cumulative bar presses on a PR schedule on second day of PR testing during infusion (Day 11). ([D-Lys3]-GHRP-6: $n = 7$; saline: $n = 8$; ghrelin: $n = 7$). * $p <$ .05.

A main effect of treatment group was found for cumulative bar presses during the PR session on day 14 of infusion ($F_{(2,19)} = 5.404$, $p = .014$). Fischer's LSD post-hoc analysis revealed that rats infused with ghrelin exhibited higher levels of bar pressing across the test session, relative to the [D-Lys3]-GHRP-6 treated rats ($p = .004$). Rats that received saline did not differ from either ghrelin ($p = .124$) or the [D-Lys3]-GHRP-6 treated rats ($p = .793$), with their average number of responses falling between the other two groups (see Figure 18).

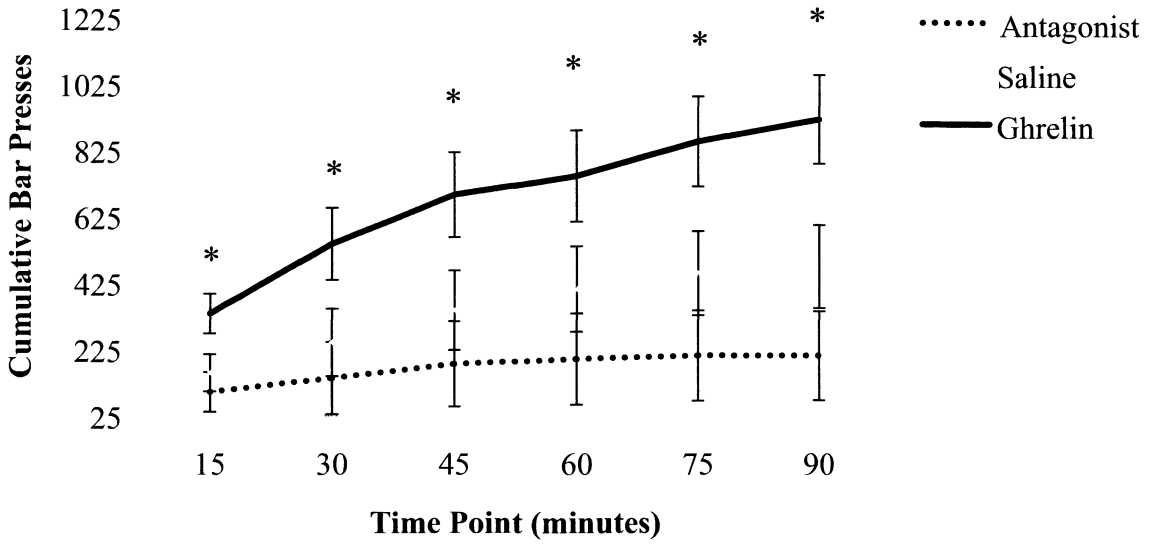


Figure 18. Mean (+/- SEM) cumulative bar presses on a PR schedule on the third day of PR testing during infusion (Day 14). ([D-Lys3]-GHRP-6: $n = 7$; saline: $n = 8$; ghrelin: $n = 7$). * $p <$ .05.

With regards to breakpoint, no differences were found between groups on Day 8 ($F_{(2,19)}$ = .832, p = ..449) or Day 11 of testing ($F_{(2,23)}$ = 1.456, p = ..256). However, during the PR session on Day 14, differences were evident between groups ($F_{(2,23)}$ = 7.398, p = .004), with the ghrelin infused rats showing an increase in effort expended to obtain chocolate-flavoured pellets relative to the [D-Lys3]-GHRP-6 group (p = .001). Ghrelin treated rats pressed the reinforcing lever 167 times on average to receive one chocolate pellet, whereas [D-Lys3]-GHRP-6 treated rats pressed the lever only 42.5 times on average to receive a similar reward (see Figure 19), demonstrating the importance of functional GHSR receptor binding for the motivation to obtain highly palatable food sources.

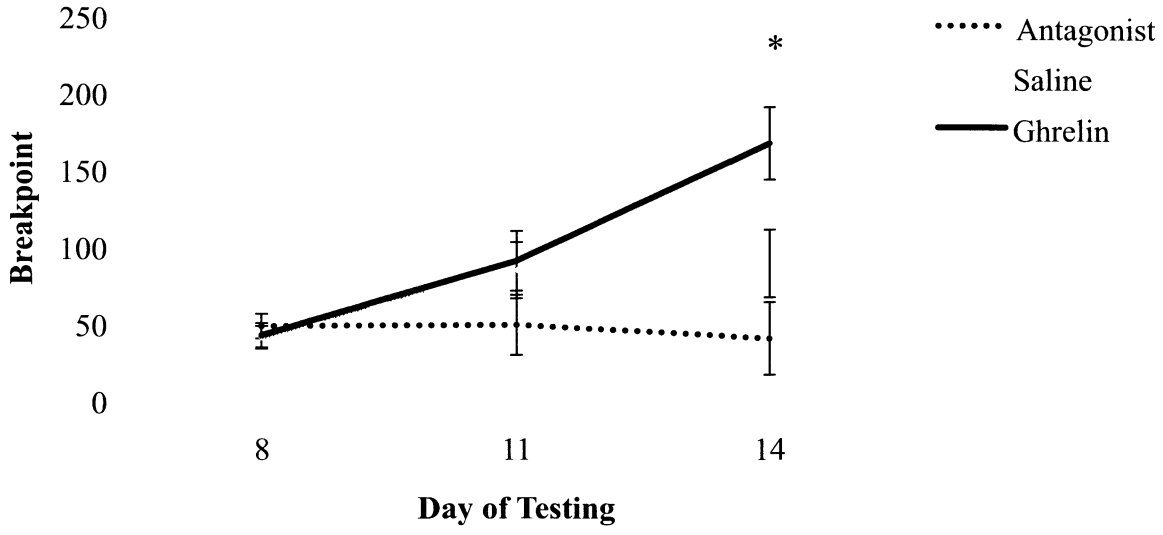


Figure 19. Mean (+/- SEM) breakpoints across each of the three PR testing days. ([D-Lys3]-GHRP-6: $n = 7$; saline: $n = 8$; ghrelin: $n = 7$). * $p < .05$.

Discussion

In the present series of studies, pharmacological manipulations were used to alter the efficacy of ghrelin binding to the GHSR receptor at the level of the VTA in order to determine the effects on feeding and its hedonic/rewarding components. The VTA is a key site in the mesolimbic dopamine system and is essential for the transmission of dopaminergic signals to target sites such as the striatum and frontal cortices; areas important for mediating the expression of motivated and/or goal-directed behaviours. Chronic two-week administration of ghrelin within this region was hypothesized to increase the intake of regular chow in a free feeding paradigm, increase the amount of calories derived from high-fat diet when given free-choice, as well as to increase the motivation to obtain a highly-palatable food source (i.e. chocolate-flavoured pellets).

In accordance with previous studies that examined the effects of *acute* ghrelin infusion on feeding responses (Naleid et al., 2005), we provide evidence that *chronic* central action of ghrelin in the VTA is also important for both *ad libitum* food consumption and body weight gain. Rats chronically treated for 14 days with ghrelin (3 nM/day or 10 nM/day) showed a dose-dependent increase in the amount of chow they consumed daily, relative to their baseline intake. However, the 10 nM dose was most effective at increasing food intake, as well as a concomitant increase in body weight. Rats treated with 10 nM ghrelin ate 7 grams more chow than their baseline on average per day during treatment, compared to saline treated rats, which ate only 3 grams more on average per day. The group treated with 3 nM of ghrelin displayed an intermediate but non-significant increase in chow intake of 4.5 grams more than baseline. Additionally, receiving 10 nM ghrelin induced an average daily body weight gain of 7.2 grams per day, whereas the saline and 3 nM ghrelin groups gained 5.9 and 6.1 grams per day on average, respectively. When translated into total weight gain over the infusion period, rats given

the high dose of ghrelin gained nearly 20 percent more over the two-week infusion period than the saline treated rats, and 15 percent more than the low ghrelin group.

These results demonstrate that exogenous ghrelin administered into the VTA can influence *ad libitum* food intake and body weight gain, a function traditionally attributed to the action of ghrelin at hypothalamic nuclei that regulate energy homeostasis (Cowley, 2003). Even in the absence of caloric restriction, chronically elevated ghrelin can serve to induce a sustained feeding response when administered in the VTA, an effect potentially mediated by it's stimulation of the mesolimbic system.

In the second experiment, which explored the intra-VTA effects of chronic ghrelin on macronutrient diet preference, we gave rats either saline, 10 nM of ghrelin/day, or a selective GHSR antagonist, [D-Lys3]-GHRP-6 (200 nM/day), to block the effects of endogenous ghrelin and determined the influence of each on the rats choice of diet. This particular antagonist has been shown to reduce both food intake and body weight gain (Asakawa et al., 2003). We hypothesized that ghrelin would increase the proportion of daily calories derived from a high-fat diet, based on findings from previous research showing that ICV ghrelin increased the choice of a high-fat option when presented with both high-fat and high-carbohydrate diets simultaneously (Shimbara et al., 2004). Alternatively, we hypothesized that ghrelin antagonism would block this effect.

The chronic blockade of ghrelin using [D-Lys3]-GHRP-6 resulted in a 10 percent reduction in the intake of high-fat diet during the treatment period when presented alongside both high-protein and high-carbohydrate diet options *ad libitum* in the home cage. Rats treated with 10 nM of ghrelin for the same period displayed a 17 percent increase in consumption of high-fat

diet, and saline controls displayed a 23 percent increase. Antagonism of the GHSR via [D-Lys3]-GHRP-6 attenuated the preference for calories derived from high-fat, compared to both saline and ghrelin groups. Caloric intake of both high-protein and high-carbohydrate diets did not differ between groups during the baseline or the post-treatment periods. However, during the treatment period saline treated rats appeared to derive a higher number of daily calories from the high-carbohydrate diet, although the percentage of total calories derived from this diet daily was very minimal compared to the other diets.

In the two final studies, the motivation to obtain chocolate flavoured pellets was assessed. Since ghrelin increases the transmission of DA in the NAc (Abizaid et al., 2006; Jerlhag et al., 2006), we hypothesized that an increase in the amount of instrumental responding for a reward would increase with ghrelin administration. In experiment 3, we investigated how ghrelin treated rats would respond on a FR 1 operant schedule, under which they receive one chocolate flavoured pellet for every correct press of the active lever. Rats were housed under *ad lib* feeding conditions and therefore remained in a relatively satiated state throughout the infusion period. While rats were trained under CR conditions, they were given free access to chow during the testing phase of the experiment.

During infusion, ghrelin treated rats exhibited greater total weight gain, weighing an average of 29 grams more than saline treated rats by the end of the infusion period. In addition, rats receiving ghrelin averaged a greater amount of chow intake daily, relative to saline controls. Further, with regard to operant responding under *ad lib* conditions, ghrelin treated rats displayed a 30 percent increase in their baseline responding on an FR 1 schedule and saline controls responded at only 60 percent of their baseline rate during training. Importantly, these rats were not only tested during the light cycle, at a time when they would not usually eat and/or forage for

food, but also in the absence of caloric restriction. If ghrelin acts within the VTA to potentiate responding for food under these conditions, it would demonstrate that under circumstances where rats are satiated, ghrelin maintains its effectiveness in promoting appetite.

In the final experiment, we used a progressive ratio schedule to assess the motivation to obtain chocolate flavoured pellets. Designed to assess how much effort an animal is willing to expend to obtain a given reinforcer, the progressive ratio schedule has been traditionally used in studies to evaluate the efficacy of pharmacological agents (Richardson & Roberts, 1996). However, it can also be applied to other natural reinforcers to examine their influence on both appetitive and consummatory behaviours. In this case we used an exponential series to assess the ability of chronic ghrelin to increase motivation to obtain chocolate flavoured pellets, under which the contingency to receive a reinforcement increased incrementally after each completed ratio. We hypothesized that the administration of [D-Lys3]-GHRP-6 would hinder instrumental responses for chocolate pellets and that chronic ghrelin infusion would potentiate responding on a PR schedule. In this study, rats were both trained and tested on a calorie restricted feeding regimen.

Analysis of cumulative bar presses during the unlimited duration test session showed that all rats displayed similar amounts of cumulative bar pressing during their first PR test on day 8 of infusion. On day 11 of infusion, [D-Lys3]-GHRP-6 treated rats began to show a reduction in PR responding overall compared to both saline and ghrelin treated rats, and ceased responding at an earlier point in the session. Ghrelin and saline treated rats both exhibited an upward trend in responding. During the final PR test session on day 14 of infusion, ghrelin treated rats exhibited a significantly greater number of bar presses at each time interval relative to the [D-Lys3]-

GHRP-6 treated rats. Saline treated rats did not differ significantly from either the ghrelin treated rats or [D-Lys3]-GHRP-6 treated rats.

The most commonly used measure of motivation obtained from a PR schedule is the breakpoint. The higher the breakpoint, the higher the cost-benefit ratio becomes, suggesting that the animal perceives the reinforcer to be increasingly salient and is willing to work harder to obtain it. In this study, breakpoints were calculated for each test session. On days 8 and 11 of testing, no significant differences were found between treatment groups. However, by day 14 of infusion, ghrelin treated rats showed an increase in breakpoint compared to the saline treated rats. [D-Lys3]-GHRP-6 treated rats again displayed the lowest levels of responding of the three groups, and by the end of the final session, were responding at a lower rate than recorded in their previous sessions. As such, it appears that the sustained elimination of ghrelin signaling specifically within the VTA can attenuate the effectiveness of endogenous ghrelin that is present as a result of CR. Conversely, the chronic stimulation of the GHSR by exogenous ghrelin can further potentiate responding under a PR schedule, even when endogenous levels of ghrelin are physiologically elevated due to CR. When the rat has to work for a reward, it seems that administration of ghrelin can potentiate the motivation to obtain it, however, when presented with highly-palatable food *ad lib*, the additional infusion of ghrelin may not be as effective in inducing an increase in the consumption of such food sources.

The current series of experiments provides evidence that chronic ghrelin not only acts centrally, but more specifically in the VTA, serving to increase food consumption and weight gain under free feeding conditions. In addition, daily caloric intake of a high-fat diet is blunted when GHSR functioning is blocked using the ghrelin antagonist, [D-Lys3]-GHRP-6. When chocolate flavoured pellets are provided in an operant chamber, ghrelin treated rats will bar press

more frequently for them on an FR1 schedule when not calorie-restricted, as compared to when they are hungry. Finally, on a progressive ratio schedule, ghrelin treated rats show an increase in effort expended to obtain the pellets after 14 days of infusion, as measured by breakpoint, whereas [D-Lys3]-GHRP-6 treatment blocked this effect. Taken together, these findings support the more recent notion that the central actions of ghrelin can occur in extra-hypothalamic regions to promote appetite, in addition to confirming previously accepted theories regarding ghrelin's orexigenic properties.

Previous studies have shown that acute ghrelin, given both systemically and centrally, serves to induce robust feeding responses and increases in body weight, as well as a decrease in energy expenditure (Lawrence et al., 2002; Tschop et al, 2002; Tschop et al;, 2000; Wren et al., 2000; Wren et al. 2001; Naleid et al., 2005). The current studies also demonstrate that ghrelin influences feeding behaviour by elevating food consumption and increasing weight gain, in accordance with this early work. However, ours is one of the first to show these effects after *chronic* central VTA ghrelin administration, not only on free-feeding behaviours, but also on the hedonic and motivational components of feeding.

An additional finding in this series of experiments was that the presence of ghrelin increased the motivation to obtain palatable food. Conversely, blockade of GHSRs in the VTA reduced the effort rats would put forth to obtain chocolate pellets. This demonstrates clearly that in the absence of ghrelin the motivation to obtain food is reduced. Further, we found that the presence of ghrelin in the VTA increased the choice of highly palatable foods, an effect demonstrated previously with acute ICV injections of ghrelin (Shimbara et al., 2004). In the current study, rats treated with ghrelin demonstrated a preference for the high fat diet over other macronutrient selections, an effect that was not observed following the administration of [D-

Lys3]-GHRP-6. These findings reveal a dual role for ghrelin acting in the VTA under chronic conditions: 1) an increase in food consumption in general and 2) an increase in the consumption of and motivation to obtain high fat and/or highly palatable foods. In humans, consumption of food high in fat has been shown to cause a smaller reduction in circulating ghrelin levels compared to both protein and carbohydrate consumption (Foster-Schubert et al., 2008). Therefore, if ghrelin levels increase preference for a high-fat diet, and ghrelin levels subsequently remain elevated, this could potentially lead to a cycle within which chronic overeating and accompanying weight gain ensue.

The role of the VTA in the modulation of feeding is complex, and appears to be closely linked to the motivational aspects of feeding, in addition to influencing the hunger/satiety pathway that is generally governed by the hypothalamus. Both regions appear to play a role in an energy regulatory system that provides peptidergic feedback from the periphery (e.g. leptin and ghrelin) to the central nervous system that is essential for the maintenance of a healthy body weight. Hypothalamic nuclei that express receptors for peripherally synthesized peptides such as leptin and ghrelin are known to work in tandem, and are key regulators of energy balance. Infusion of ghrelin into the hypothalamus increases food consumption via activation of NPY cells (Baskin et al., 1999; Olszewski et al., 2003), whereas leptin administration activates POMC cells that inhibit NPY and subsequent feeding (Traebert et al., 2002). However, the VTA also expresses a dense population of receptors for both ghrelin and leptin (Zigman et al., 2006), suggesting that metabolic signals can act directly on regions within reward circuitry. As reciprocal connections between hypothalamic nuclei and the VTA exist, it is likely that the action of ghrelin in the VTA operates in parallel with hypothalamic networks that regulate energy homeostasis to modulate the perception of food reward.

The mesolimbic dopamine pathway is an evolutionarily important circuitry that developed to increase the drive for animals to engage in behaviours that are important for survival, such as feeding and sexual behaviour. Within this pathway, the VTA provides the main source of dopamine to the NAc via efferent projections. The NAc subsequently sends projections to other regions of the brain, including the PFC and amygdala to influence motivated behaviours. The ability of increased DA transmission in the NAc to promote the exhibition of goal-directed behaviours has been studied extensively. Dopamine signaling in the NAc has been shown to influence feeding, drug-self administration, sexual behaviour, and even maternal responsiveness in rats (Volkow & Wise, 2005; for maternal behavior review see Numan, 2006). If ghrelin acts on the dopamine system to influence reward, it is plausible that intra-VTA ghrelin could increase the incentive salience or motivational properties of particularly appetizing foods, resulting in a preference for them (Perello et al., 2009), as well as a greater effort to obtain them.

In addition to the influence that ghrelin has on the motivation to obtain chocolate flavoured pellets, it is possible that ghrelin acting in the VTA serves to enhance other motivated behaviours, not only for natural reinforcers, such as food and sex, but also artificial rewards such as drugs of abuse. Previous reports have demonstrated that animals housed under caloric restriction, causing an elevation of endogenous ghrelin levels, show an increase in the locomotor effects observed following administration of various drugs of abuse (Carr et al., 2003). CPP for drug rewards such as cocaine are also increased after systemic ghrelin treatment (Davis, Wellman & Clifford, 2007). Since sexual behaviour is also heavily influenced by the DA system (for review see Melis & Argiolas, 1995), studying the effects of ghrelin on sexual behaviour may help in understanding whether or not the effects of ghrelin on the motivation to obtain food

reward can be generalized to all goal-directed behaviours that use dopamine as their main neurostransmitter.

The neuronal plasticity demonstrated by rapid synaptic rearrangement of inputs onto DA neurons in the VTA that have been stimulated with ghrelin in vitro could serve as a potential mechanism underlying the behavioural effects of ghrelin on motivated behaviours. These DA neurons exhibit an increase in the ratio of glutamatergic to GABAergic inputs, ultimately leading to an increase in DA transmission downstream in the NAc (Abizaid et al., 2006). It is tempting to speculate that the increase in breakpoint observed by day 14 of infusion may have resulted from a plastic change, resulting in an increase in excitation of DA neurons in the VTA. The fact that we did not see an immediate effect of ghrelin treatment suggests that it may take time for this synaptic plasticity to translate into a significant change in behaviour. Recently, one group has shown that acute ghrelin administration can cause an increase in operant responding for a high-fat diet in mice (Perello et al., 2009), although ghrelin in this study was given systemically, resulting in a more varied distribution of the ghrelin that gains access to the CNS. We show here that ghrelin or its antagonist in the VTA specifically can induce such behavioural changes.

This study showed that circulating factors such as ghrelin can affect reward pathways under conditions during which feeding is not metabolically necessary. Chronic ghrelin infused into the VTA increased the motivation for food and the energy expended to obtain it, even when hunger was not the primary motivating factor. Further, the effects of ghrelin on food consumption and weight gain, as well as specifically inducing a preference for high-energy foods with high fat content, could potentially result in an unhealthy increase in energy consumption. Combined with an increase in sensitivity to highly palatable food sources and reduction in energy expenditure, this may provide a basis for the development of an obese phenotype.

The actions of ghrelin on the GHSR in the VTA have also been implicated in the regulation of mood and depression (Nestler & Carlezon Jr., 2006). Chronic social defeat stress, which can result in a depressive phenotype (e.g. anhedonia), causes a significant increase in VTA DA neuron firing (Krishnan, Han, & Graham, 2007). In addition, the elevated ghrelin levels observed after such a chronic stress paradigm can result in an increase in excitatory potential of DA neurons in the VTA, as well as an increase in DA release downstream in the NAc (Abizaid et al., 2006; Jerlhag et al., 2007). This has been shown to result in a decrease in depressive-like behaviours, as measured by the forced-swim test (Lutter, Sakata, & Osbourne-Lawerence, 2008). Considering the current results, ghrelin may play a role in mediating the effects of stress-induced depression by reducing the likelihood of anhedonia. This could result in an increase in the exhibition of motivated responding for positive reinforcers as a potential coping mechanism to deal with stressors.

While the current behavioural results point to the importance of ghrelin for both *ad libitum* food intake and the motivation to obtain highly-palatable food, the possibility that the mechanism of action responsible for such effects are dependent on altered dopaminergic functioning was not directly assessed. We can only speculate here that, as ghrelin's action in the VTA causes a downstream influx of DA into the NAc (Jerlhag et al., 2006), particularly in the shell region (Quarta et al., 2009), it results in an augmentation of instrumental responses required to obtain an appetizing food source, in the absence of necessity. In the future, it will be important to examine the complexity of this pathway more closely, possibly using pharmacological methods to illustrate an interaction between ghrelin and dopamine function.

Evolutionarily, the actions of the DA system on the motivation to attend to and obtain reinforcers (e.g. palatable food) makes intuitive sense in an environment where extensive

foraging and food hoarding would provide an adaptive advantage. However, due to the current "obesigenic" environment, the adaptive value of this system is greatly reduced, whereas the average BMI of individuals worldwide has increased substantially. Research has shown that many parallels exist between addiction to pleasurable (i.e. dopamine releasing) stimuli such as drugs of abuse, and food. If the two phenomena share neural underpinnings it may suggest that one can actually become addicted to highly-palatable foods, which in today's environment is likely to increase the prevalence of obesity.

As chronic overeating and the resultant obesity that accompanies it has become an increasing threat to the health of individuals worldwide, it is important to determine what can be done to reduce it. As we have shown, antagonism of ghrelin not only reduced body weight gain and preference for a high-fat diet when no effort was required, it also blunted the effect of ghrelin on the effort expended to obtain highly-palatable foods. Emerging evidence, including the current study, suggests that acute CNS administration of a class of novel GHSR antagonists has anorexigenic effects in rats (Salome et al., 2009). This provides a basis from which to pursue the potential therapeutic benefits of such pharmacological interventions for the treatment of obesity.

Appendix A

Table 1. Macronutrient composition for operant pellets (45mg)

Purified Chocolate Pellets	Nutritional Content	% by weight
	Protein	18.8
	Carbohydrates	61.5
	Fat	5.0
	Fiber	4.6
	Ash	4.4
	Moisture	< 5
Total kcal/gram		3.68

Appendix B

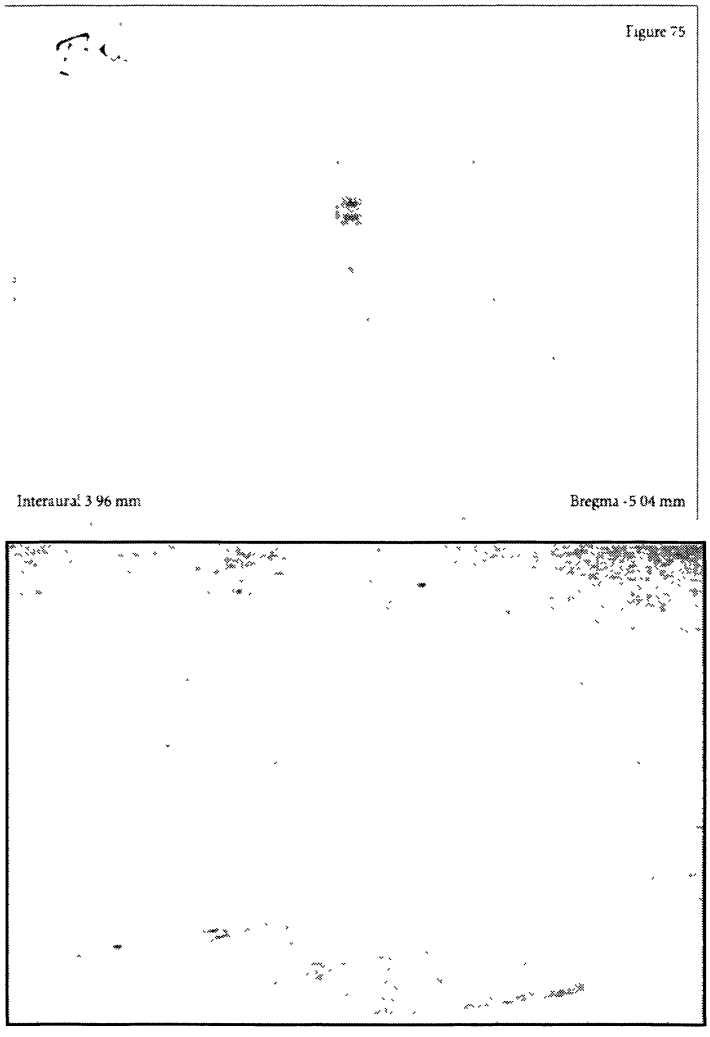
Table 2. Macronutrient composition of diets used in experiment 2

Diet	Macronutrient	% by weight	% kcal from
Fat (60%)	Protein	23.5	18.4
	Carbohydrates	27.3	21.3
	Fat	34.3	60.3
Total kcal/gram	5.1		
Carbohydrates (70%)	Protein	17.7	17.8
	Carbohydrates	70	70.4
	Fat	5.2	11.8
Total kcal/gram	4		
Protein (60%)	Protein	60	64.7
	Carbohydrates	21.1	22.7
	Fat	5.2	12.6
Total kcal/gram	3.7		

Appendix C

Table 2. Cannula placement accuracy by study

Study	Total # of Cannulations	Incorrect Placements
1	24	3
2	24	2
3	20	3
4	24	2



Representative photomicrograph showing a unilateral VTA cannula placement and accompanying rat brain atlas reference plate (adapted from Paxinos & Watson, 2005).

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