Role of Ghrelin in Modulating the Motivational Components of Reproduction

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A thesis submitted to
The Faculty of Graduate and Postdoctoral Affairs
In partial fulfillment of the requirements of the degree of
Master of Science
In Neuroscience

Carleton University
Ottawa, Canada

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Abstract

Reproductive fitness and physiology are tightly coupled with, and dependent upon, metabolic needs and energy balance. Ghrelin, an orexigenic peptide, has been shown to play an inhibitory role on overall reproductive physiology. In contrast, ghrelin plays a modulatory role in motivated reward-driven behaviors through its actions on the dopaminergic mesolimbic pathway. The main objective of the current thesis was to investigate the effects of ghrelin acting on the medial preoptic area (mPOA) and the ventral tegmental area (VTA) on the anticipatory and consummatory aspects of male sexual behaviour. Experiment 1 investigated the behavioral effects of ghrelin infusions directly into the mPOA when compared to saline in the presence of a sexually-experienced receptive female. Experiment 2 examined the effects of intra-VTA ghrelin, ghrelin receptor (growth hormone secretagogue; GHSR) antagonist, and saline on several aspects of male sexual behaviour under different dietary manipulations. Results from the first experiment suggest that ghrelin acts on the mPOA to inhibit sexual appetitive behaviours and shorten copulatory behaviours without influencing food consumption. In the second experiment, GHSR antagonism reduces anticipatory behaviour when compared to saline. Furthermore, food deprived males displayed decreased anticipatory behaviors when compared to ad libitum male rats. In sum, ghrelin and its receptor play a modulatory role in sexual behaviors in the male rat, and this effect is dependent on the brain area targeted. The current research supports the idea that the availability of oxidizable fuels, and its subsequent neurochemical signals, may set the stage for the quantities and sensitivity of several neuropeptides and neurotransmitters acting on critical brain regions involved in appetitive behaviors.

Acknowledgements

I would like to express my sincerest gratitude to my mentor, Dr. Alfonso Abizaid for all of his guidance, patience, and wisdom. I am so grateful for all of his encouragement and for believing in me. I have come out of this degree a better writer, speaker and scientist and it is all thanks to him. I would also like to thank Dr. Barbara Woodside for all the great discussion and input. Furthermore I would like to thank my amazing family, Mom, Dad, Josh, Michelle, Chafic, Louisette and Zeke for their continued love and support. Lastly, I would like to thank my best friends, Karine and Alex, for helping me so much whenever I needed it. I could not have done this without you, thank you all.

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* $p < .05$, ** $p < .01$

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List of Abbreviations

ACTH, Adrenocorticotropin hormone; AgRP, agouti-related protein; AL, ad libitum; ARC, arcuate nucleus of the hypothalamus; CNS, central nervous system; CRH, corticotropin releasing hormone; DA, dopamine; DHT, dihydrotestosterone; EB, estradiol benzoate; FD, food deprived; FSH, follicle stimulating hormone; HPA, hypothalamic pituitary adrenal axis; HPG, Hypothalamic pituitary gonadal axis; GH, growth hormone; GHSR, growth hormone secretagogue receptor; GNRH, gonadotropin releasing hormone; GOAT, ghrelin o-acyltransferase; LH, luteinizing hormone; LM, lordosis magnitude; MC, melanocortin; mPFC, medial prefrontal cortex; mPOA, medial pre-optic area; NO, nitric oxide; NOS, nitric oxide synthase; NPY, neuropeptide Y; NA, Nucleus accumbens; NaCl, sodium chloride; OVX, ovariectomy; P, progesterone; PAG, periaqueductal gray; PFA, paraformaldehyde; POMC, proopiomelanocortin; PVN, paraventricular nucleus of the hypothalamus; SNS, sympathetic nervous system; VMH, ventral medial nucleus of the hypothalamus; VNO, vomeronasal organ, VTA, ventral tegmental area

1.Introduction

Reproductive fitness and physiology are tightly coupled with, and dependent upon, energy balance and metabolism. In states of negative energy balance, vertebrates will prioritize survival and the consumption of food, rather than seek a potential mate. However, well-nourished states and overall health promote sexual motivation and its subsequent reproductive success. The physiology underlying energy metabolism and reproductive success exhibit a reciprocal relationship (Schneider, 2004). For instance, several orexigenic peptides have been shown to exert inhibitory reproductive effects at the level of hypothalamic pituitary gonadal (HPG) axis such as agouti-related protein (AgRP), Neuropeptide Y (NPY), Orexin, and Galanin-like peptides (Ammar et al., 2000; Gundlach, 2002; Pu et al., 1998; Schioth et al., 2001). Conversely, several peptides that decrease food intake such as cholecystokinin and α -melanocyte stimulating hormone, stimulate the release of luteinizing hormone in the HPG axis (Alde & Celis, 1980; Kimura et al., 1983; Perera et al, 1993). In sum, peptides that control metabolic and nutritional status also play an essential role in reproduction.

The focus of this thesis will be on ghrelin; a potent peripheral peptide signaling energy insufficiency (Zigman and Elmquist, 2003). Ghrelin plays a critical role in energy homeostasis, along with the motivational (appetitive) and consummatory behaviors of food intake. This orexigenic peptide has been shown to play an inhibitory role in the HPG axis (Fernandez-Fernandez et al., 2004). Extensive investigations have been conducted on the role of ghrelin in reproductive processes that are sensitive to metabolic states such as pregnancy, gonadal development and puberty, although little research has been

conducted on ghrelin's role in hormone-dependent sexual behaviors (Kawamura et al., 2003; Fernandez-Fernandez et al, 2005).

1.1 Ghrelin

Ghrelin is a primarily gut-derived orexigenic peptide that regulates food intake, body weight and energy homeostasis (Figure 1). Ghrelin was first isolated in the X/A endocrine cells of the stomach, but is also secreted by cells in the small intestine, brain, heart, kidney, gonads, pancreas, lung, placenta and lymphocytes (Ariyasu et al., 2001; Cowley et al, 2003; Tena-Sempere, 2008; Ueberberg et al., 2009). Ghrelin is synthesized as a preprohormone and is further processed via alternative splicing into proghrelin. The inactive proghrelin or des-acyl ghrelin is abundant in the plasma (Murakami et al., 2002). An enzyme known as ghrelin o-acyltransferase (GOAT) post-translationally modifies des-acyl ghrelin into its active, octanoylated form known as acyl-ghrelin (Yang et al., 2008).

Acylated ghrelin is the only known endogenous agonist for the growth hormone secretagogue receptor (GHSR), a metabotropic Gq-protein coupled receptor (Kojima, 1999). There have been two forms of the GHSR receptor identified: the functionally active GHSR-1a, along with the truncated form of this receptor GHSR-1b that is devoid of ghrelin signaling capabilities (Van der Lely et al., 2004). Acylated ghrelin binding to its functional receptor causes the increase of intracellular calcium, through either an adenylate cyclase-protein kinase A pathway, or phospholipase C/ protein kinase C pathway, depending on whether it's acting on the arcuate nucleus (ARC) or acting on growth hormone (GH) release (Kohno et al., 2003; Chen et al., 1996). Type 1a GHSR has been found to be widespread in peripheral tissues such as the stomach, pancreas,

adrenals, testies, ovary, thyroid, and adipose tissue suggesting a diverse repertoire of endocrine and non-endocrine related biological functions (Zigman, Jones, Lee, Saper, & Elmquist, 2006; Papotti et al, 2000).

Ghrelin receptors have also been identified in the central nervous system (CNS), and are expressed in abundance in areas involved in homeostatic feeding such as the hypothalamus, and particularly the arcuate nucleus (ARC) and the paraventricular nucleus of the hypothalamus (PVN; Howard et al, 1996, Guan et al, 1997; Katayama et al, 2000). Interestingly, ghrelin receptors are also located in extra-hypothalamic sites, such as the ventral tegmental area (VTA), hippocampus, substantia nigra, raphe nuclei and the laterodorsal tegmentum (Guan et al., 1997, Katayama et al, 2000; Abizaid 2006). The expression of GHSR in these extra-hypothalamic sites suggests that ghrelin may have a role in processes other than homeostatic and metabolic functions.

1.2 Hypothalamic Regulation of Food Intake and Energy Balance

Ghrelin is secreted in response to states of energy insufficiency; gastric secretion of ghrelin rises in response to a prolonged period of fasting or in anticipation of a meal, and rapidly declines after a meal is consumed (Cummings et al., 2001; Tschop, Smiley & Heiman 2000; Sanchez et al., 2004). In rats, both peripheral or central ghrelin administration evoke a rapid feeding response (Asakawa, et al., 2001; Wren et al., 2000). Ghrelin exerts its orexigenic effects by targeting brain areas implicated in food intake and appetite, including the ARC, PVN, lateral hypothalamus and VTA (Nakazato et al., 2001).

1.3 Ghrelin and Food Reward

In addition to the hypothalamus, ghrelin targets a number of extrahypothalamic regions that are important in the regulation of motivated behaviors including the motivation to eat independently of energy status (Richardson & Gratton 1998, Zigman et al., 2006; Abizaid et al., 2006; Skibicka et al., 2011). These include the VTA; a region of the brain that contains predominantly dopamine neurons project to the limbic forebrain structures including the Nucleus accumbens (NA), medial prefrontal cortex (mPFC), hippocampus, and amygdala (Heimer et al., 1997). Evidence supports the idea that ghrelin enhances the rewarding properties of food by directly stimulating the mesolimbic DA pathway and may do so in the absence of metabolic imbalance .

Ghrelin exerts its effects on reward by acting directly on the dopaminergic projections in the mesocorticolimbic system. GHSR is present on both the cells bodies and presynaptic afferents of DA neurons originating from the VTA and projecting to the NA (Guan et al, 1997; Abizaid, 2006). The presence of GHSR in the VTA is imperative for ghrelin-induced food intake and DA release in the NA. Pharmacologically blocking GHSR in the VTA, blocks both ghrelin-induced food intake along and accumbal DA release (Abizaid et al, 2006; Jerlhag et al., 2007; Jerlhag et al., 2011). Studies in rodents show that both central and peripheral ghrelin administration lead to an increase in DA release and turnover in the NA (Jerlhag et al., 2007; Abizaid et al., 2006). In addition, ghrelin has been shown to increase the sensitivity and firing rate of DA projections to the NA by increasing the relative number of excitatory versus inhibitory inputs on their perikarya (Abizaid et al., 2006). In sum, ghrelin works to increase the probability and frequency of DA output from VTA neurons, and this effect is mediated through the

GHSR.

Ghrelin's role in the mesolimbic dopamine circuit appears to reinforce the palatability, consumption and rewarding value of food intake in both mice and rats (Disse et al, 2010; Nakazato et al., 2011; Skibicka et al., 2011; Perello, 2010; Wren et al., 2001). These effects are mediated by the actions of ghrelin on the mesolimbic dopaminergic pathway, in particular projections originating from the VTA into the NA. Furthermore, when ghrelin is administered directly into the VTA it stimulates food intake (Naleid, Grace, Cummings, & Levine, 2005; Egecioglu, et al., 2010, Abizaid et al., 2006), along with motivation to seek a highly palatable reward as measured by progressive ratio responding under restricted feeding conditions (King, Isaacs, O'Farrell, & Abizaid, 2011). Predictably, administration of a GHSR antagonist directly into the VTA blocks ghrelin-induced food intake (Naleid et al., 2005; Abizaid et al., 2006) and incentive motivation behavior for a sucrose reward, the latter assessed by a PR operant paradigm (Skibicka, Hansson, Alvarez-Crespo, Friberg & Dickson, 2011). In addition, pharmacological inhibition of, or genetically knocking out, the GHSR has been shown to abolish conditioned place preference for highly palatable foods (Perello et al., 2010; Egecioglu et al., 2010). In summary, it is clear that ghrelin plays an important role in motivation and food reward as shown by increased feeding when administered directly into components of the mesolimbic DA reward pathway.

1.4 Ghrelin and Drug Reward

The mesolimbic DA pathway is implicated in reward seeking behaviors other than food, including drugs. Consistent patterns of behavior can be seen in both drug addicts

and obese people in regards to their lack of inhibition and disruption in reward systems (Holden, 2001; Potenza et al, 2003). Dietary manipulations such as food deprivation or food restriction, which correlate with a rise in endogenous levels of ghrelin, have been shown to enhance drug seeking, self-administration and behavioral locomotor effects of psychostimulants in rodents suggesting a potential role for ghrelin in drug addiction (Guialillo, 2002; Dickson et al., 2011; Tessari 2007).

Ghrelin administration has been shown to amplify behavioral responses to, and rewarding value of ,certain addictive recreational drugs such as cocaine, amphetamines, opiates, alcohol and nicotine (Dickson et al., 2011; Davis 2007; Tessari 2007; Wellman, Davis and Nation, 2005). For example, the locomotor stimulating effects of psychostimulants as well as the formation of a place preference for a psychostimulant-associated environment are potentiated following ghrelin administration in rodents (Davis 2007, Tessari 2007, Wellman et al., 2005; Abizaid et al., 2011). Conversely, ghrelin receptor antagonism attenuates these psychostimulant-induced effects (Jerlhag, 2010) and also decreases dopaminergic output to the NAcc (Jerlhag 2010). In mice, genetic knock-out of ghrelin attenuates locomotor responses, along with blunted sensitization effects to cocaine administration (Abizaid, 2011). Taken together, ghrelin has been shown to play a modulatory role in motivated reward-driven behaviors through its actions on the dopaminergic mesolimbic pathway.

1.5 Ghrelin and Sexual Behavior

Given that ghrelin modulates food and drug motivation and reward, it is possible that it also regulates sexual behavior through its actions on the dopaminergic system and

the HPG. What is universally accepted is that ghrelin has an overall inhibitory role on the HPG axis at the level of gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH) and gonadal steroidogenesis (Tena Sempere, 2007). In female rats, peripheral, central or ICV administration of ghrelin results in a suppression of LH secretion (Fernandez-Fernandez et al., 2004; Fernandez-Fernandez et al., 2006; Forbes, Li, Kinsey-Jones & O'Byrne, 2009). Similarly, in males, ghrelin results in suppression at the level of LH secretion in juvenile, adult and gonadectomized animals (Fernandez-Fernandez et al., 2004). Moreover, ghrelin administration in male rats has been shown to lower testicular testosterone secretion in a dose dependent manner, although this was only demonstrated *in vitro* (Tena-Sempere et al., 2002).

Behaviorally, negative states of energy balance have been shown to dampen appetitive sexual behaviours, without affecting the consummatory behaviors (Klingerman & al., 2011; Sachs, 1965, Shah & Nyby, 2010). Certain androgen-dependent pre-copulatory behaviors in male house mice, such as inter-male aggression and emission of courting-like ultra-sonic calls, have been shown to be suppressed by peripheral ghrelin administration while other behaviors e.g. preference for female cage bedding such as (Shah & Nyby, 2010). Other studies have demonstrated that the onset of sexual behavior in male rats is delayed in those undergoing substantial food deprivation relative to ad libitum, which would be associated with an increase in circulating ghrelin concentrations although male consummatory sexual behavior is not impaired by food deprivation once the mating begins (Sachs, 1965). Overall, there is a gap in the literature pertaining to the behavioral and mechanistic effects of ghrelin on male sexual behavior.

The regulation of male sexual behavior is highly dependent on circulating levels of

hormones. Early studies involving castrated males show the importance of the presence of an intact HPG axis, along with steroidal output from the gonads. In gonadectomized male rats, testosterone replacement has been shown to restore, as well as maintain male sexual behavior (Grunt and Young, 1952). Levels of testosterone are critical in males to for the display of sexual behaviors. Testosterone works as a prohormone; primarily mediating its effects through its biproducts such as dihydrotestosterone (DHT) and estrogen. Testosterone can be reduced via 5-alpha-reductase to DHT and act through an androgenic pathways or be aromatized to estrogen and act through an estrogenic pathway. Testosterone's conversion to DHT appears to mediate penile reflexes and tactile sensitivity (Gray et al., 1980; Meisel et al., 1984) whereas the estrogenic pathway appears to mediate most copulatory behaviors in male rodents (Christensen and Clemens, 1975; Davidson, 1969). In particular, it appears that aromatization to estrogen is critical for testosterone's central actions on male rodent sexual behavior. Pharmacological inhibition of this aromatase enzyme greatly impairs male consummatory behaviors as measured by frequency and latency of mounts, intromissions and ejaculations over time (Roselli, Cross, Poonyagariyagorn, & Stadelman, 2003).

1.6 Pathways of Male Sexual Behavior

Male sexual behavior consists of both anticipatory and consummatory behaviors. Appetitive behaviors can be measured in a variety of ways depending on the experimental environment. Some examples of appetitive measures include: ability and latency to overcome an obstacle to gain access to a receptive female (Obstruction test; Plaud and Martini, 1999), locomotor activity observed in a conditioned anticipatory period before exposure to a receptive female, as well as the latency from the time of

introduction to a female until the first mount (Mendelson & Pfaus, 1989). Sexual motivation can be calibrated by the amount of work the male has to put in to obtain access to a receptive female and can be assessed with certain parameters such as bar-pressing to gain access to a receptive female, or as previously stated, an obstruction test.

Anticipatory behavior and reinforcement of sexual motivation is primarily mediated by the mesolimbic system. Specifically, DA release in the NA increases during the anticipatory phase of male sexual behavior (Pfaus et al., 1990). DA agonists, such as L-DOPA, facilitated sexual excitement and sexual behaviors in the castrated male rat (Malmnas, 1976; Malmnas, 1977). Haloperidol, an inverse DA agonist, decreases anticipatory level change behavior when administered directly into the NA without significantly affecting male consummatory behavior (Pfaus & Phillips, 1991). Furthermore, a decrease in mesolimbic DA activity, induced by activation of DA autoreceptors in the VTA, had an overall dampening effect on the general sensorimotor responsiveness to copulation and motivational stimuli (Hull et al., 1991). Considering ghrelin and its receptor have been shown to enhance of DA-ergic output from the VTA to the NA, this suggests a possible role for ghrelin in sexual motivation and appetitive behaviors.

The medial pre-optic area (mPOA) is the predominant structure mediating male sexual behavior. The mPOA integrates environmental and internal physiological stimuli, to give rise to the motor output needed to initiate and sustain proper male sexual behavior. Chemosensory input that provides information about the receptivity and familiarity of a potential female mate, is critical for the expression of male sexual behavior. This chemosensory pathway consists of the olfactory bulbs, vomeronasal organ (VNO), MeA

and their respective connecting tracts, and ultimately get processed, in the mPOA. The mPOA sends reciprocal connections to the VTA, brain stem, periaqueductal gray (PAG), as well as other areas of the hypothalamus such as the PVN and arcuate nuclei (Simerly & Swanson, 1988). Consummatory male sexual behavior is ablated in mPOA-lesioned rodents, but this manipulation may not have as grave of an effect on sexual motivation (Parades, 2003; Hull et al., 1997). The integrity of the mPOA along with its respective levels of steroid hormone levels, catecholamines and monoamines, are critical in mediating male sexual behavior.

DA in the mPOA has been consistently shown to facilitate male sexual behavior. DA agonists facilitate male copulatory behavior by increasing the number of ejaculations, along with decreasing the latency to ejaculate (Hull, Bitran, Pehek, Warner, Band & Holmes, 1986). Whereas, both neurotoxic lesions and direct microinjection of DA antagonists into the mPOA impair copulation and genital reflexes, thereby increasing ejaculation latencies, and decreasing the number of ejaculations per session relative to controls (Bitran, Hull, Holmes & Lookingland, 1988; Pehek et al., 1988). Several factors modulate the release of DA in the mPOA, and ultimately influence sexual activity, such as testosterone and nitric oxide (NO; Dominguez & Hull, 2005).

The integration and interpretation of olfactory, tactile and auditory sensory cues in the mPOA is sensitive to changes in hormonal milieu (Hull, Du, Lorrain, & Matuszewich, 1997). The mPOA has been shown to be a target in the androgen-induced enhancement of male sexual behavior. Testosterone in the mPOA of the male rat works to modulate neural responsiveness to olfactory cues (Pfaff & Pfaffmann, 1969). Moreover, testosterone in the mPOA has the ability to further modulate levels of neurotransmitter

release. Testosterone has been shown to upregulate nitric oxide synthase (NOS), which increases levels of NO, thereby increasing DA release in the mPOA of male rats potentially by inhibiting reuptake or reversing the DA transporter (Hull et al., 1997; Kiss, J.P., Hennings, Zsilla & Vizi, 1999; Lorrain, Matuszewich, Howard, Du, & Hull, 1996; West, Galoway, & Grace, 2002). In sum, factors such as testosterone and NO act in the mPOA to affect DA release and its actions on copulatory behavior.

Ghrelin binding, and ghrelin-immunoreactivity has been demonstrated in the male rat mPOA, although it's behavioral or physiological function in this area is still largely unknown (Guan & al, 2007; Weidmer & al., 2011; Zigman & al, 2006). In female rats, peripheral ghrelin administration down-regulates Kisspeptin (Kiss1) expression in the mPOA, but had no effect on Kiss1 in the ARC (Forbes, Li, Kinsey-Jones & O'Bryne, 2009). A downregulation of Kiss1 in these neurons has been shown to correlate to a suppression of pulsatile LH secretion in the female rat (Kinsey-Jones & al., 2009).

Further studies are required to investigate the potential role of ghrelin, and its receptors in the mPOA of male rats. Considering there is ghrelin-induced activation of these mPOA neurons in the rat (Weidmer et al., 2011) ghrelin may have a potential role in mediating sexual behavior.

1.7 Aims and Hypothesis

Research conducted for my Master's thesis investigated the effects of ghrelin acting on essential brain regions implicated in male sexual behavior, such as the hypothalamus and the mesolimbic system. Therefore two experiments were carried out to explore the effects of ghrelin acting on both the mPOA and the VTA in modulating male anticipatory and consummatory sex behaviors. The first experiment consisted of giving central

microinjections of ghrelin or saline directly into the mPOA. Considering ghrelin has an overall inhibitory role on the HPG axis and reproduction, it was hypothesized that ghrelin may inhibit the display of certain male appetitive and consummatory sexual behaviors. In the second experiment, the role of ghrelin on sexual behavior was investigated by administering central microinjections of ghrelin, GHSR antagonist or saline directly into the VTA. Central administration of ghrelin into the VTA directly has been shown to increase overall motivation and rewarding value for a variety of rewards such as food and drugs. Given that as ghrelin has an overall excitatory role on the DA cells of the mesocorticolimbic pathway, it was hypothesized that ghrelin may enhance the display of certain male anticipatory sexual behaviors.

2. Materials & Methods

2.1 Experiment 1: Ghrelin administration bilaterally into the MPOA of male rats

Animals

Thirty-three sexually naive Long-Evans male rats, weighing 150-200g, were obtained from Charles River (St-Constant, Quebec). Males were housed in groups of 4 in large plexiglass chambers dimensions with betachip bedding on a reversed lighting schedule (12/12 h light-dark, with lights off at 8pm). Food and water were given ad libitum. Post-surgery, males were individually housed in shoebox cages dimensions, made from. A group of 30 female Long-Evans rats (200-250g) were used as stimulus animals. Female rats were housed in pairs in shoebox cages and all other housing conditions were identical to those described for males. These sexually-experienced stimulus females were primed with estradiol benzoate (EB; 10 µg/0.1mL sesame oil) and progesterone (P; 500 µg/0.1mL sesame oil) administered 48 and 4 hours prior to sexual

training respectively. All experiments were conducted following the Canadian Council on Animal Care (CCAC) guidelines, and were approved by the Local Ethics Committee at Carleton University.

Ovariectomy Surgery

One week after arrival, female rats were bilaterally ovariectomized (OVX) through lumbar incisions under a mixture of 4 parts ketamine hydrochloride (100mg/ml) to 3 parts xylazine hydrochloride (20mg/ml) administered by intraperitoneal injection (1ml/kg of body weight). Females were treated post-operatively with subcutaneous injections of 3cc physiological saline, and Metacam (0.1 ml).

Cannulation Surgery

After the 6th and final training session, bilateral cannulae were implanted into all males under isofluorane anaesthetic. All bilateral cannulae, dummy blocker cannula and infusion cannula were obtained from Plastics One (Roanoke, VA). A bilateral 22-gauge stainless steel guide cannula were implanted 1 mm above the mPOA, using the following coordinates from bregma on a flat skull: AP -0.6 , ML ± 0.5 , DV -7.0 mm. Both dummy blocker cannula and infusion cannulae were 28-gauge thickness, and were 1 mm longer than the guide cannulae. Animals were given a subcutaneous injection of Metacam (1mg/kg) to provide analgesia on recovery from the anaesthetic. Males were given a one-week recovery period in which they were monitored closely.

Hormone and Drug Administration

All steroid compounds were received from Steraloids (Newport, RI). EB(10 μ g) and P (500 μ g) were dissolved in 0.1mL sesame oil under low heat for approximately 30 minutes, and stored at room temperature. Females were primed with subcutaneous

injections of EB 48 hours before, and P 4 hours prior to each of the training and testing sessions with males. Ghrelin and ghrelin receptor antagonist (D-Lys3-GHRP-6) were obtained from Peptides International, and were mixed in physiological saline (0.9% NaCl) at doses of 1µg/µl, and 10µg/µl, respectively.

Ghrelin and saline were infused into the MPOA at a rate of 0.5µl/min per side for one minute using a Harvard Apparatus infusion pump, for a total volume of 1µl. The infusion cannula remained in place for one minute after completion of drug infusion to ensure that the entire drug was absorbed. The infuser was then removed, the dummy cannula was replaced, and the male was immediately placed in the bilevel chamber.

Behavioral Measures

All sexual behavior training and testing occurred in bi-level chambers (Mendelson & Pfaus, 1989), during the middle third of the dark cycle. These chambers are designed to facilitate the experimenter's view of the full behavioral repertoire of sexual behaviors (Mendelson & Pfaus, 1989; Pfaus, Smith, Coopersmith, 1999). Males are placed in the bottom compartment of the bi-level chamber for a 10 minute habituation period. Next, females are placed on the top level for a 30-minute test. Behaviors were video-recorded with a Sony Handycam, and subsequently scored using the Behavioral Observation Program customized for rodent sexual behavior (Cabilio, S., unpublished computerized event recorder)

Anticipatory and consumatory behaviors are recorded and scored for both males and females. For males, anticipatory behavior was defined as the number of level changes during the 10-minute habituation period in the bilevel chamber (Mendelson and Gorzalka, 1987). Consumatory behavior was coded as the number of mounts, intromissions and

ejaculations (Pfaus et al., 1999). Latencies were also recorded for first time to mount, inter-intromission intervals (III), ejaculations and post-ejaculatory refractory periods.

Female proceptive behaviors were coded as solicitations; defined as head-wise orientation towards the male followed by a run-away to the same or a different level, and hops and darts. Female receptive sexual behavior were measured by the degree of lordosis, which is defined as a reflexive arching of the back as a result of flank stimulation. Lordosis magnitude (LM) were measured on a 4-point scale according to Hardy and Debold (1972) such that no lordosis was coded as a zero, and increasing magnitudes from low to high were coded from 1-3.

Food intake after the experimental session was also recorded. Males were not given access to food or water during their 40-minute behavioral testing session in the bilevel chambers. Once males are placed in their home cage after their experimental session, they had access to 300g of rat chow for 24 hours post-testing. Food intake was recorded after 1 hour, 2 hours, and 24 hours post-testing.

Experimental Design and Procedure

Males were given 6 training sessions with sexually experienced females prior to surgery. To investigate whether the cannulation surgery would have any consequence on male sexual behavior, the final training session was recorded and behavior was scored as a pre-operative baseline. After the final training session, the males underwent bilateral cannulation surgery as described above. After a 7-day post-operative recovery period, all experimental males received a 0.1 µl injection of physiological saline and were given a 30-minute copulatory session with hormonally primed females to create a baseline for their post-operative sexual behavior.

The effect of intra mPOA ghrelin administration on male sexual behavior was evaluated using a within subjects design. After the saline baseline test, males were assigned to one of two cohorts ($n = 12$ animals/cohort). One cohort received ghrelin microinjections on the first test, and saline microinjections on the second test. The second cohort received these treatments in the opposite order; saline on test 1 and ghrelin on test 2. All males received ad libitum food and water before receiving ghrelin administration or saline administration. Testing for sexual behavior always took place in the middle of the dark cycle, when rodents are most active.

After receiving all treatments, the males were subjected to a final baseline test to investigate whether repeated microinjections had an effect on their sexual behavior. One day after final baseline test, males were then sacrificed and transcardially perfused with 4% paraformaldehyde (PFA) to prepare tissue for checking cannula placements.

2.2 Experiment 2: Ghrelin and GHSR antagonist administration unilaterally into the VTA of male rats

Animals

Twenty-four sexually naive Long-Evans male rats, weighing 150-200g, were obtained from Charles River (St-Constant, Quebec). The housing conditions of the males and females, hormone and drug administration, ovariectomy surgery and behavioral measure for Experiment 2 are all equivalent to Experiment 1.

Surgery

Males underwent unilateral cannulation surgery into the VTA with the coordinates AP -5.3 mm, ML $+2.0$ mm, DV -7.6 mm; Paxinos and Watson, 1998. A unilateral cannula was chosen because it has been previously shown that the

administration of a ghrelin or ghrelin receptor antagonist unilaterally into the VTA is sufficient to enhance operant behaviours aimed at obtaining a sucrose or drug reward (Skibicka, Hansson, Alvarez-Crespo, Friberg & Dickson, 2011) The surgical procedure is identical to that of experiment 1. All of the cannula equipment was obtained from Plastics One (Roanoke, VA): Guide cannula were 10mm below pedestal, dummy cannula and internal cannula extended 1mm below the guide cannula.

Experimental Design and Procedure

Presurgical treatment i as described for Experiment 1 above. Males then underwent unilateral cannulation surgery into the VTA and given a 7-day recovery period. A post-operative baseline of male sexual behaviors was measured after a 1µl injection of 0.9% physiological saline. Half of the rats had access to ad-libitum food, while the other half of the group, were food deprived for 16 hours before testing. The first group ($n=12$) group receiving ad libitum access to food and received either 1µg/µl of ghrelin or saline in each of the two experimental tests. The second group ($n=12$) receiving acute food deprivation was either given 10µg/µl of GHSR antagonist, or 1 µl of saline in each of the two experimental tests. Animals received each of their respective drug and control treatments, in a counterbalanced order. A final baseline test was done where animals received 0.9% saline prior to experimental session. The following day animals were given 1µl of cresyl violet solution and transcardially perfused to prepare tissue for cannula placement verification.

2.3 Statistical Analyses

A mixed factorial ANOVA was used to analyze all behavioral data from the experimental sessions using SPSS software (Version 18), and following a significant

difference between groups, pairwise comparisons will examine differences between group means. The independent variables are the drug treatment groups and the dietary regime. The dependent variable is male anticipatory and consummatory behaviour. The level of significance was set at 0.05 for all comparisons.

3. Results

3.1 Experiment 1: Medial Pre-Optic Area

Nine out of the 33 males used either did not recover from surgery or survive through all of the 2 baseline tests and 3 experimental tests, and were thus sacrificed and their data removed from the analysis. Data from a further 5 animals were removed because of incorrect cannula placement.

Anticipatory Behavior

All means and standard errors ($M \pm SE$) are represented in Table 1. There were no significant differences seen in anticipatory behavior during the first 5-minute interval between ghrelin (15.58 ± 1.45) and saline (18.26 ± 1.26) microinjected animals, $F_{(1,18)} = 3.208$, $p = .090$, $\eta_p^2 = .151$. During the full 10-minute anticipatory period, males microinjected with ghrelin (22.68 ± 1.59) displayed lower levels of anticipatory behaviors when compared to saline (29.42 ± 1.51), $F_{(1,18)} = 23.965$, $p = .001$, $\eta_p^2 = .571$ (See Figure 2).

Consummatory behavior

There were no significant differences in the number of mounts and latency between mounts in males who received ghrelin when compared to saline, $F_{(1,18)} = 1.10$, $p = .345$, $\eta_p^2 = .064$ and $F_{(1,18)} = .303$, $p = .539$, $\eta_p^2 = .024$, respectively. Furthermore, no significant differences were seen in the number and latency of intromissions within-

groups, $F_{(1,18)} = .565, p = .573, \eta_p^2 = .032$ and $F_{(1,18)} = .105, p = .750, \eta_p^2 = .006$, respectively. There were no significant differences in number of ejaculations between the ghrelin and saline groups, $F_{(1,18)} = 1.659, p = .215, \eta_p^2 = .089$. Males receiving ghrelin microinjections displayed a shorter latency to ejaculate (437.10 ± 17.93), when compared to saline (493.91 ± 21.57), $F_{(1,18)} = 5.617, p = .029, \eta_p^2 = .238$ (see Figure 3). There were no significant differences in refractory periods post ejaculation between ghrelin and saline infused males, $F_{(1,18)} = 1.066, p = .317, \eta_p^2 = .062$. Finally, there were no significant differences in the number of intromissions required for the first ejaculation between groups, $F_{(1,18)} = 2.050, p = .169, \eta_p^2 = .273$. For all means and standard errors, refer to Table 2.

Female Behavior

Females copulating with males microinjected with ghrelin and saline displayed similar levels of appetitive behaviors such as hops/darts and solicitations, $F_{(1,18)} = .150, p = .861, \eta_p^2 = .009$ and $F_{(1,18)} = .362, p = .698, \eta_p^2 = .022$ respectively (Figure 4). No significant differences were seen in lordosis ratio (LR) or female level changes between females copulating with ghrelin and saline males, $F_{(1,18)} = .324, p = .576, \eta_p^2 = .018$ and $F_{(1,18)} = .110, p = .744, \eta_p^2 = .006$ respectively. For all means and standard errors, refer to Table 3.

Food Consumption Post-Test

No significant differences in food intake were seen between treatment groups at either 1-, 2-, or 24-hours post test: $F_{(1,18)} = .447, p = .512, \eta_p^2 = .024$, $F_{(1,18)} = 2.312, p = .146, \eta_p^2 = .114$ and 24-Hour $F_{(1,18)} = 1.975, p = .177, \eta_p^2 = .099$, respectively (Figure 5). For all means and standard errors, refer to Table 4.

3.2 Experiment 2: Ventral Tegmental Area

Twenty-one out of the 24 males recovered from surgery, and survived all experimental sessions. The data from four animals were removed from the analysis because of incorrect cannula placement.

Anticipatory Behavior

Ad libitum (AL) males displayed significantly more level changes ($M = 16.70$, $SE = 1.04$) during the first 5-minutes of the anticipatory period when compared to the Food deprived (FD) group ($M = 11.50$, $SE = .99$), $F_{(1,16)} = 12.642$, $p = .003$, $\eta_p^2 = .441$. No significant within-group effects of drug were seen in AL and FD groups, $F_{(1,9)} = 3.361$, $p = .100$, $\eta_p^2 = .272$ and $F_{(1,7)} = 2.605$, $p = .151$, $\eta_p^2 = .151$ respectively (see Figure 6).

Males in the AL group displayed significantly more anticipatory behaviors (30.30 ± 1.53), as measured by level changes during a 10-minute interval, than FD males (19.00 ± 1.66), $F_{(1,16)} = 24.904$, $p = .000$, $\eta_p^2 = .609$. Furthermore, males which received ghrelin did not significantly differ from when they received saline on an AL schedule, $F_{(1,9)} = .027$, $p = .873$, $\eta_p^2 = .003$. In the FD group antagonist administration significantly reduced anticipatory behavior ($M = 15.38$, $SE = 2.47$) when compared to saline under a FD dietary schedule ($M = 22.62$, $SE = 1.71$), $F_{(1,7)} = 7.519$, $p = .024$, $\eta_p^2 = .518$. For all means and standard errors, refer to Table 5.

Male Consummatory Behavior

AL and FD males did not differ significantly on any measure of consummatory behavior. There were no significant differences in the number of mounts or the latency to mount between AL and FD males, $F_{(1,15)} = .537$, $p = .479$, $\eta_p^2 = .034$ and $F_{(1,15)} = 3.242$, $p = .092$, $\eta_p^2 = .178$ respectively. Furthermore, there were no significant differences in the

number, or latency to intromit between AD and FD males, $F_{(1,15)} = 2.326, p = .148, \eta_p^2 = .134$ and $F_{(1,15)} = .332, p = .573, \eta_p^2 = .022$ respectively. No significant differences were found between number of ejaculations or latency to ejaculate, $F_{(1,15)} = 1.954, p = .182, \eta_p^2 = .115$ and $F_{(1,15)} = 1.176, p = .297, \eta_p^2 = .077$ respectively. No significant difference between refractory period, male level changes, and interintromission interval, $F_{(1,15)} = .062, p = .807, \eta_p^2 = .004, F_{(1,15)} = .01, p = .923, \eta_p^2 = .001$, and $F_{(1,15)} = .003, p = .958, \eta_p^2 = .000$ (Figure 7). For all means and standard errors, refer to Table 6.

Female Behavior

Females that copulated with AL males displayed significantly more hops and darts than females copulating with FD males, $F_{(1,15)} = 4.953, p = .042, \eta_p^2 = .248$. There were no significant between-groups differences in females copulating with AD and FD males overall, $F_{(1,8)} = 1.191, p = .307, \eta_p^2 = .13$ and $F_{(1,7)} = .71, p = .474, \eta_p^2 = .075$, respectively. There were no significant between-group differences in the number of solicitations in females mating with AD males or FD males, $F_{(1,15)} = .746, p = .401, \eta_p^2 = .047$. No significant differences within-group differences in the number of solicitations from females copulating with AD or FD males, $F_{(1,8)} = 7.557, p = .025, \eta_p^2 = .486$ and $F_{(1,7)} = .243, p = .637, \eta_p^2 = .034$ respectively. No significant differences in LR, Hit rate, female level changes, all $p > .10$ (Figure 8). For all means and standard errors, refer to Table 7.

Food Consumption Post-Test

Males on FD ate significantly more than AL animals at 1-, 2-, and 24- Hours post testing: $F_{(1,15)} = 34.786, p = .000, \eta_p^2 = .699, F_{(1,15)} = 15.093, p = .001, \eta_p^2 = .502$ and $F_{(1,15)} = 4.401, p = .053, \eta_p^2 = .227$ respectively. No significant differences within groups

was seen at 1-, 2-, and 24- hours, $p > .05$ for all (Figure 9). For all means and standard errors, refer to Table 8.

Discussion

Experiment 1

The purpose of the current study was to investigate the role of ghrelin on the display of both anticipatory and consummatory male sexual behaviors. Given that ghrelin has an overall inhibitory role on the HPG axis and reproduction, it was hypothesized that ghrelin would inhibit the display of certain male appetitive and consummatory sexual behaviors. In experiment 1, both ghrelin and saline were infused in a counterbalanced order directly into the mPOA, a critical region mediating male sexual behaviors. Overall, we observed that intra-mPOA ghrelin (1) caused a decrease in the level of anticipation, and (2) shortened ejaculation latency without affecting food intake, when compared to saline controls. The anticipatory results from the current study are consistent with Schneider's (2004) theory that the mechanisms underlying both energy balance and reproduction are reciprocally linked. Schneider (2004) hypothesizes that during mild energetic challenges, sexual motivation is inhibited, without affecting the whole HPG axis.

Ghrelin binding and immunoreactivity has been observed previously in the rat mPOA (Weidmer et al., 2011; Zigman et al, 2006) but the precise function of ghrelin receptor signalling in this area has yet to be elucidated. Little is known about the type of neurons that contain GHSR in the mPOA and how ghrelin may affect its function in reproduction and temperature regulation. A recent study has discovered GHSR on GnRH neurons in the mPOA (Farkas, Vastagh, Sarvari & Liposits, 2013). Farkas et al. (2013)

found that ghrelin acts in a GHSR-mediated manner to decrease firing of GnRH neurons, and this process further involves both estrogen and retrograde cannabinoid signaling in females. Seeing as GnRH neurons are at the core of the reproductive axis and steroid hormone production, any ghrelin-induced modulation of this system may affect multiple aspects of male reproductive behaviors. However, the current study uses an acute microinjection of ghrelin as well as its antagonist, and both of these substances have a relatively short half-life. Therefore, their actions on the HPG, and their respective influence on sex steroid production, are an unlikely explanation of the immediate results obtained. Indeed, the effect of hormones is substantially more intricate and time consuming than those of neurotransmitters.

Ghrelin may be dampening anticipatory behavior through its interactions with a variety of neurotransmitters. Prior to the present research, much work had been conducted on the interplay between ghrelin and opiates in the mesolimbic system, specifically its role in mediating the rewarding value of both regular and palatable food in rats (Kawahara et al., 2013). In general, male ejaculation induces a release of opioid activity in mPOA and is sustained throughout the post-ejaculatory interval (Szechtman, Hershkowitz & Simantov, 1981). B-endorphin, a cleavage product of POMC, binds preferentially to μ opioid receptors, which are known to play a role in male sexual behaviour and are abundant in the mPOA (Coolen, Fitzgerald, & Lehman, 2004). Intra-mPOA administration of a μ opioid receptor agonist substantially reduces male sexual behavior, elucidating the inhibitory role that opioids play (Band & Hull, 1990; Hughes, Everitt, & Herbert, 1987; Matuszewich et al., 1995). If ghrelin is interacting with μ and subsequently increasing opioid activity in the mPOA - this may explain the decrease in

anticipatory behaviors seen in the current study.

The second significant result derived from the current study is that ghrelin in the mPOA decreases the latency for males to attain their first ejaculation when compared to their control saline group. Contrary to our hypothesis, ghrelin had a facilitative, rather than inhibitory, effect on consummatory behavior when administered into the hypothalamus. In response to signals of energy insufficiency, the observed decrease in the latency of ejaculation may be reflective of an increase in mating efficiency, in order to allocate more time to feeding or food seeking behavior.

While elucidating the underlying mechanism of ghrelin's role in decreasing ejaculation latency is beyond the scope of this project, we suggest that it might be acting on the sympathetic nervous system (SNS) as well as neurotransmitter release. Firstly, ghrelin may be influencing ejaculation latencies through an interaction with serotonin, a neurotransmitter that has been associated with states of satiety and sexual inhibition (Meyerson, 1960). The mPOA receives serotonergic projections from the raphe nucleus in the brain stem, which serves to enhance GnRH secretion (Gouveia and Franci, 2004). Infusions of serotonin directly into the mPOA or NAcc of male rats substantially prolongs ejaculation latencies (Fernandez-Guasti, Escaalante, Ahlenius, Hillegaart & Larsson, 1992). Conversely, decreasing serotonin levels with a 5-HT_{1A} autoreceptor agonist shortened the latency for ejaculation (Ahlenius et al., 1981). The relationship between ghrelin and the central serotonergic system is reciprocal (Hansson et al., 2013). For instance, ghrelin decreases serotonin release from hypothalamic synaptosomes (Brunetti et al, 2002). Taken together, ghrelin may be working to decrease serotonin levels in the mPOA, which would in turn shorten ejaculation latencies.

Furthermore, DA is another neurotransmitter that has been consistently shown to serve a critical role in the mPOA, mediating male consummatory sexual behaviour. DA agonists in the mPOA facilitate male copulatory behavior by increasing the number of ejaculations, whereas DA antagonists impair copulation and genital reflexes along with decreasing the latency to ejaculate (Bitran, Hull, Holmes & Lookingland, 1988; Hull, Bitran, Pehek et al., 1988; Pehek, Warner, Band & Holmes, 1986). Ghrelin may be modulating intra-mPOA DA levels through two potential mechanisms. Firstly, by acting through the nitric oxide (NO) pathway to ultimately increase dopamine, and drive sympathetically mediated ejaculation. As has been previously reported by Gaskin et al. (2003), ghrelin upregulates nitric oxide synthase (NOS) in the hypothalamus, increases levels of NO, and consequently, DA levels. Increasing intra-mPOA DA above a certain threshold is imperative for the shift from D1 to D2 receptor activation, which is necessary for promoting ejaculation (Hull et al., 1989). Secondly, GHSR and dopamine receptor D2 (DRD2) have been found to form heteromers in hypothalamic neurons (Kern, Albarran-Zeckler, Walsh, & Smith, 2012). In sum, ghrelin may be up regulating NOS, or acting directly through its GHSR1a:DRD2 to mediate mPOA DA, enhancing sympathetically-mediated ejaculatory mechanisms.

A third potential mechanism of action involves the SNS, which is an important player in the emission stage of ejaculation (Birowo et al, 2010). Ghrelin increases sympathetic tone through the enhancement of hypothalamic-pituitary-adrenal axis activity (HPA; Asakawa et al, 2001; Giordano et al, 2006). Previous data suggests that ghrelin activates both corticotropin releasing hormone (CRH) and vasopressin release, consequently facilitating adrenocorticotropin hormone release (ACTH; Korbontis et al.,

2004). CRH axons have been shown to synapse with GnRH neurons in the mPOA (Li et al., 2010). If ghrelin is increasing overall sympathetic tone, this may help explain the facilitated ejaculation that is observed in ghrelin-treated animals.

The current investigation demonstrated that ghrelin in the mPOA did not increase food intake. This is an intriguing observation considering that ghrelin acts in an orexigenic manner in other areas of the hypothalamus, such as the ARC and PVN. This finding also lends support to the idea that the infused drug was contained rather well in the specific area of interest, rather than diffusing into other areas of the hypothalamus.

In sum, when comparing intra-mPOA ghrelin-infused animals with saline-infused animals, the former displayed less activity in anticipation to a receptive female, as measured by level changes. Further, males receiving ghrelin displayed shorter latencies to their first ejaculation as compared to saline animals. Finally, no significant difference in food intake was seen 1-, 2-, or 24-hours post-testing across all groups, suggesting that ghrelin in the mPOA has no effect on food consumption. Consequently, ghrelin may be acting on the mPOA to inhibit sexual appetitive behaviours and shorten copulatory behaviours without influencing food consumption.

Experiment 2

Experiment 2 examined the effects of infusions of ghrelin and GHSR antagonist directly into the VTA on precopulatory and consummatory aspects of male sexual behavior. Central administration of ghrelin into the VTA increases overall motivation and rewarding value for rewards such as food and drugs (Abizaid et al, 2006; Abizaid et al., 2011; Davis 2007; Jerlhag et al., 2007; Jerlhag et al., 2011; Tessari 2007, Wellman et al., 2005). Seeing as ghrelin has an overall excitatory role on the DA cells of the

mesocorticolimbic pathway, it was hypothesized that ghrelin enhances the display of male anticipatory sexual behaviors. There were two main goals of Experiment 2; (1) to investigate effects of ghrelin and GHSR antagonist administered directly into the VTA and (2) to explore the effects of food deprivation and ad libitum access to food on male sexual behaviour.

i) Ghrelin and GHSR antagonist administration into the VTA

Ghrelin increases mesolimbic DA, which is critical for driving male anticipatory sexual behaviors (Pfaus et al., 1990). Thus, any alteration in this system should affect anticipatory behaviors, without having a significant impact on consummatory behaviors. In the current study, intra-VTA infusions of GHSR antagonist decreased anticipatory behavior when compared to their FD saline counterparts. It is known that administration of a GHSR antagonist decreases DA release in the mesolimbic pathway and is most likely the underlying mechanism contributing to this effect (Abizaid et al, 2006; Jerlhag et al., 2007; Jerlhag et al., 2011). This is consistent with previous data showing that giving a GHSR antagonist decreases motivation and reward-seeking behaviors in rats (Jerlhag, 2010). These behavioral results are also consistent with the finding that administration of Haloperidol, an inverse DA agonist, directly into the mesolimbic system decreases anticipatory level change behavior without significantly affecting male consummatory behavior (Pfaus & Phillips, 1991). This is the first real evidence supporting a role for the GHSR receptor in the anticipatory aspects of male sexual behaviour.

Ghrelin administration into the VTA was hypothesized to increase male anticipatory behaviors, while the GHSR antagonist was shown decrease such behaviors. Unexpectedly, ghrelin-infused animals did not increase their anticipatory behavior when

compared to the saline infused rats as hypothesized. The most feasible explanation for this outcome is that the visual and olfactory cues conditioned with the bi-level chamber were very strong, exerting a substantial effect on anticipatory behavior in each condition. Both the ghrelin and saline groups displayed a large number of level change behavior during the 10-minute anticipatory period. It is possible that upon microinfusion and placement into chamber, levels of dopamine peaked in both of these conditions, creating a ceiling effect. Consequently, the effect of ghrelin on this system may have been negligible given that the animals were already in a heightened state of anticipation.

ii) Effects of food deprivation on male sexual behavior

Consistent with our hypothesis, results illustrated that a dietary manipulation such as a 16-hour FD had a substantial dampening effect on the anticipatory behavior of male rats, without affecting consummatory behavior. These findings are parallel to earlier work by Sachs (1965) in which he found that FD delayed the onset of sexual behavior in male rats, although male consummatory sexual behavior is not impaired by FD once the mating begins. A similar phenomenon is observed in male Syrian hamsters where food restriction decreases only appetitive, and not consummatory aspects of male sexual behaviour (Lazarini et al., 1988; Schneider et al, 1988). As ghrelin is secreted during FD - a state of negative energy balance - it may be working in the mPOA to dampen sexual motivation based on findings from experiment 1.

The decrease in male anticipatory behaviors in the FD group may be due to decreases in GnRH neuronal activity, LH release or steroid hormone production, as ghrelin has been shown to decrease all of these (Farkas et al., 2013; Fernandez-Fernandez et al, 2006). The mPOA is critical for the integration and interpretation of olfactory and

auditory sensory cues, and its activity is very sensitive to changes in the hormonal milieu (Hull, Du, Lorrain, & Matuszewich, 1997). Steroid hormones such as estrogen, progestins and androgens prime the neural circuitry necessary for the processing of secondary reinforcers such as the scent of a receptive female (Kow & Pfaff, 1988; Pfau, Kippin, Coria-Avila, 2003). Being steroid sensitive in nature, a ghrelin-induced decrease in steroid hormone levels in the mPOA would, in effect, account for a decrease in responsiveness to sexual stimuli.

As previously mentioned, in a FD state, ghrelin levels increase and target the hypothalamus to promote the orexigenic NPY/AgRP pathways (Gualillo et al., 2002; Nakazato et al., 2001). The orexigenic peptides AgRP and NPY act to inhibit cellular and behavioral reproductive processes (Cowley et al., 2003; Spinedi et al., 2006). Intraventricular administration of NPY, for instance, has been previously shown to suppress sexual motivation in the male rat, and greatly inhibit sexual behavior (Clark, Kalra & Kalra, 1997). Overall, orexigenic peptides such as NPY and AgRP affect rewarding behaviours, and tend to have an inhibitory role on the reproductive behaviors and their success (Altizer & Davison, 1999; Tracey et al., 2008).

Moreover in a FD state, ghrelin promotes appetite through indirect inhibition of POMC neurons by increasing the number of inhibitory inputs on these cells, and therefore decreasing release of anorectic α -melanocyte stimulating hormone (α -MSH) into the PVN (Cowley et al, 2003). In addition, ghrelin stimulates AgRP release, which acts to block the binding of anorectic α -MSH to its melanocortin (MC) receptors (MC3 and MC4; Cowley et al., 2001; Ollman et al., 1997). Administration of the MC agonist bremelanotide increases appetitive (level changes), penile erection and consummatory

behavior in male rats (Pfaus, Giuliano, & Gelez, 2007; Pfaus, 2009; Wessells, 2003). Bremelanotide infusions directly into the mPOA enhances proceptive behavior in the female rat; this effect is blocked by the co-administration of a MC-4 receptor antagonist (Pfaus, Giuliano, & Gelez, 2007). The potential mechanism by which MC affects the mPOA is through interaction with DA presynaptic terminals to increase DA release (Pfaus, Giuliano, & Gelez, 2007). The decrease in sexual behaviors seen during an FD state may be partially explained by the inhibition of MC, which normally promotes sexual behaviors and LH secretion (Alde & Celis, 1980; Pfaus, Giuliano, & Gelez, 2007; Pfaus, 2009; Wessells, 2003).

A final explanation for the decrease in sexual appetitive behaviors in an FD state may be due to a decrease in circulating levels of sex hormones. Ghrelin decreases GnRH and LH secretion, which reduces circulating levels of testosterone. Testosterone and estrogen are important for male rat sexual behaviour, both for the consummatory behavior and for priming the responsiveness to sexual incentives and cues (Clancy, Zumpe & Michael, 2000; Parsons, Rainbow, Pfaff, & McEwen, 1982; Roselli et al., 2003; Sachs & Meisel, 1994). Testosterone has been shown to modulate neuronal responsiveness to olfactory cues such as estrous females, particularly in the mPOA (Pfaff & Pfaffman, 1969). Therefore if FD is decreasing levels of overall circulating testosterone, this could in turn interfere with the processing of chemosensory sexually-relevant cues and subsequently decrease anticipatory behaviors.

Future Directions

Future studies should investigate the mechanisms underlying ghrelin's role on a variety of neurotransmitters and hormones mediating male sexual behavior. Firstly, it

should be considered that in the current study the male rats underwent mild food deprivation, which may not have been enough of an energetic challenge to fully view the effect of energy insufficiency on reproduction. Future studies should consider using a harsher energetic challenge such as a long food restriction schedule to bring the animals down to 90% of their weight, which is consistent with other rodent reward studies (Sedki et al., 2013).

Prospective studies should also consider repeating the current study in females. The cost of reproduction is substantially more expensive for females, and thus we can confidently hypothesize that these kinds of energetic challenges will have more profound inhibitory effects on female sexual behavior. Peripheral ghrelin administration and food restriction has been shown to inhibit female receptivity. Furthermore, this reduction is correlated with percentage of body weight loss (Bertoldi et al., 2011). Therefore central ghrelin administration should be considered in critical brain areas of female sexual behavior that contain the receptor for ghrelin, such as the mPOA, VTA and ventromedial hypothalamus (VMH; Zigman 2006).

Conclusion

Results from the present investigation demonstrate that ghrelin modulates male sexual behavior, although its role is dependent on the brain region affected. In the face of mild energetic challenges, sensitivity to neuropeptides and hormones is enhanced to dampen sexual motivation while sparing the function of the HPG axis (Klingerman et al, 2010; Schneider 2013). Intuitively, inadequate metabolic energy signals should in turn selectively focus attention on conserving fuels necessary for survival, rather than expending energy on reproduction. The current study falls within the aforementioned

paradigm; administration of intra-mPOA infusions of ghrelin decreased anticipatory behaviors and ejaculation latency in the sexually experienced male rat. The present findings also contribute a new mechanism pertaining to ghrelin's actions on male sexual behaviors.

In the second experiment, we investigated the role of ghrelin mediating sexual behavior in the mesolimbic reward pathway, as it has been previously shown to augment other rewarding behaviors (Abizaid et al, 2006; Jerlhag et al., 2007; Jerlhag et al., 2010; Jerlhag et al., 2011). Our findings indicate that GHSR-antagonist decreased anticipatory behaviour, which was consistent with previous findings that antagonism of GHSR leads to a decrease in motivation and consummation of other natural rewards and drugs of abuse (Abizaid et al, 2006; Disse et al, 2010; Jerlhag et al., 2007; Jerlhag et al., 2010; Jerlhag et al., 2011; Nakazato et al., 2011; Skibicka et al., 2011). This is the first evidence demonstrating a role for the ghrelin in VTA-mediated anticipatory sexual behavior, without exerting an effect on consummatory behaviour. Overall data suggest differential roles of ghrelin depending on whether it is acting on the mPOA or the VTA.

It must be kept in mind that the mPOA and VTA are reciprocally linked, and their patterns of catecholamine efflux during copulation are interestingly similar (Blackburn, Pfaus, & Phillips, 1992). Firstly, the mPOA receives a surplus of DA inputs from a wide variety of regions such as the zona incerta, MeA, ventrocaudal posterior hypothalamus, adjacent medial supramammillary nucleus and VTA (Miller and Lonstein, 2009; Moore, 1987; Simerly & Swanson, 1986). Projections from VTA-mPOA are primarily DAergic (Miller & Lonstein, 2009). Ghrelin administration has been shown to increase the sensitivity and firing rate of VTA DA projections to the NA, along with its subsequent

DA release and turnover (Abizaid et al., 2006; Jerlhag et al., 2007). As DA in the mPOA is critical for the expression of male sexual behaviour, the ghrelin-induced enhancement of DA tone from mesolimbic pathway may be further modulating male sexual behaviors via the VTA-mPOA pathway.

While projections from VTA-mPOA are primarily DAergic, reciprocal projections from mPOA-VTA appear to be mainly DA-sensitive GABAergic inputs. The mPOA sends dense projections to the VTA and these areas may be influencing one another (Simerly & Swanson, 1988; Tobiansky et al., 2013). The majority of the mPOA-VTA efferents are GABAergic and co-localized with DA receptors, and thus are sensitive to DA activity (Tobiansky et al., 2013). Tobiansky (2013) suggest that the GABAergic mPOA-VTA projections modulate mesolimbic DA. Moreover, the rostral portion of the mPOA is predominantly important in the appetitive aspects of sexually behaviour, whereas the caudal is more influential in consummatory aspects (Balthazart & Ball, 2007). Tobiansky (2013) demonstrated more abundant GABAergic projections to the VTA originate from rostral - rather than caudal - portions of the mPOA, therefore stressing the importance of the mPOA-VTA interplay in appetitive sexual behaviors.

In sum, the current research supports the idea that the availability of oxidizable fuels and its subsequent neurochemical signals, may set the stage for the quantities and sensitivity of several neuropeptides and neurotransmitters acting on critical brain regions involved in appetitive behaviors. Ghrelin and its receptor play a modulatory role in sexual behaviors in the male rat, and this effect depends on the brain area targeted. Consistent with previous results, this potent peripheral orexigenic signal plays an inhibitory role in the HPG axis, and its subsequent reproductive behaviors (Fernandez-Fernandez et al.,

2004). In contrast, ghrelin plays a modulatory role in motivated reward-driven behaviors through its actions on the dopaminergic mesolimbic pathway (Abizaid, 2011; Davis 2007, Tessari 2007, Wellman et al., 2005). Overall, fluctuations in metabolic state drive the switch in motivational attention between sexual and ingestive behaviors, in order to maximize reproductive successes and maintain survival.

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Table 1

Male anticipatory level changes during 5- and 10-minute intervals upon administration of ghrelin or saline bilaterally into the mPOA (mean $\pm$ SEM).

Time	Treatment	
	Ghrelin	Saline
5-Minutes	15.58 $\pm$ 1.26	18.26 $\pm$ 1.45
10-Minutes	22.68 $\pm$ 1.59	29.42 $\pm$ 1.26**

** $p < .001$ from ghrelin

Table 2

Male consummatory behavior displayed during the 30-minute copulatory testing with a receptive female (mean $\pm$ SEM). Males received bilateral microinjections of either ghrelin or saline bilaterally into the mPOA.

		Treatment	
		Ghrelin	Saline
Mount	Number	10.26 $\pm$ 2.29	8.68 $\pm$ 1.41
	Latency	322.55 $\pm$ 27.76	355.59 $\pm$ 30.81
Intromission	Number	39.37 $\pm$ 2.58	41.32 $\pm$ 1.97
	Latency	281.01 $\pm$ 9.18	285.30 $\pm$ 9.13
Ejaculation	Number	2.95 $\pm$.143	2.74 $\pm$.104
	Latency	437.10 $\pm$ 17.93	493.92 $\pm$ 21.57*
Refractory Period		343.81 $\pm$ 24.44	378.28 $\pm$ 14.92
Intros to 1 st ejaculation		13.84 $\pm$ 1.19	15.37 $\pm$.89
III		12.65 $\pm$.99	12.33 $\pm$.83
MLC		45.89 $\pm$ 3.78	50.26 $\pm$ 5.63

Interintromission interval (III) and male level changes (MLC).

* $p < .05$ from ghrelin

Table 3

Female appetitive and receptive behaviors (mean $\pm$ SEM) of females copulating with either ghrelin- or saline-infused males

	Treatment	
	Ghrelin	Saline
Hops/Darts	10.84 $\pm$ 1.57	9.58 $\pm$ 1.43
Solicitations	4.32 $\pm$.81	4.68 $\pm$.61
Lordosis Ratio	2.59 $\pm$.039	2.62 $\pm$.06
Hit Rate	.82 $\pm$.031	.839 $\pm$.02
Female Level Changes	86.47 $\pm$ 6.23	89.42 $\pm$ 5.40

Table 4

Food intake as a function of group and hours after testing (mean $\pm$ SEM)

Hourly Food Intake	Treatment	
	Ghrelin	Saline
1-Hour	5.37 $\pm$.806	4.68 $\pm$.60
2-Hours	7.16 $\pm$ 1.05	5.89 $\pm$.72
24-Hours	22.47 $\pm$ 1.69	19.37 $\pm$ 1.90

Table 5

Anticipatory behavior of males given ghrelin, saline or GHSR antagonist administered directly into the VTA (mean $\pm$ SEM).

Time	Treatment			
	Ad Libitum		16-Hour Food Deprivation	
	Ghrelin	Saline	Antagonist	Saline
5-Minutes	17.8 $\pm$ 1.26	15.6 $\pm$ 1.137	10.5 $\pm$ 1.52 [#]	12.5 $\pm$.627 [#]
10-Minutes	30.00 $\pm$ 2.65	30.60 $\pm$ 2.07	15.38 $\pm$ 2.47 [#]	22.62 $\pm$ 1.71 ^{** #}

^{**} $p < .001$ from antagonist

[#] $p < .05$ from ad libitum

Table 6

Means and standard errors of male consummatory behavior of males given ghrelin, saline or GHSR antagonist administered into the VTA (mean $\pm$ SEM).

Time		Treatment			
		Ad Libitum		16-Hour Food Deprivation	
		Ghrelin	Saline	Antagonist	Saline
Mount	Number	6.56 $\pm$ 2.01	7.22 $\pm$ 2.11	9.88 $\pm$ 2.57	6.75 $\pm$ 2.72
	Latency	266.96 $\pm$ 51.68	301.44 $\pm$ 44.32	334.59 $\pm$ 52.34	404.04 $\pm$ 48.85
Intro	Number	41.33 $\pm$ 2.95	38.00 $\pm$ 4.76	32.88 $\pm$ 3.71	33.00 $\pm$ 2.31
	Latency	256.54 $\pm$ 31.60	282.82 $\pm$ 10.62	229.51 $\pm$ 38.43	277.87 $\pm$ 16.64
Ejac	Number	3.11 $\pm$.20	3.11 $\pm$.11	3.5 $\pm$.189	3.25 $\pm$.164
	Latency	141.46 $\pm$ 17.96	126.42 $\pm$ 13.04	178.93 $\pm$ 37.66	167.93 $\pm$ 61.20
Intros to 1 st ejac		12.11 $\pm$ 1.33	11.89 $\pm$ 2.00	9.13 $\pm$ 1.42	10.88 $\pm$ 1.641
Refractory Period		347.98 $\pm$ 20.15	332.68 $\pm$ 17.37	327.36 $\pm$ 25.72	344.89 $\pm$ 23.21
Male level change		53.11 $\pm$ 6.44	49.67 $\pm$ 6.72	52.88 $\pm$ 9.78	48.25 $\pm$ 5.11
III		11.70 $\pm$ 1.39	12.42 $\pm$ 1.36	11.66 $\pm$ 1.84	12.63 $\pm$ 1.06

Table 7: *Female appetitive and consummatory sexual behaviors (mean $\pm$ SEM) of females copulating with males who received either intra-VTA ghrelin, saline or antagonist under different food regimes.*

[#] $p < .05$ from ad libitum groups

Time	Treatment			
	Ad Libitum		16-Hour Food Deprivation	
	Ghrelin	Saline	Antagonist	Saline
Hops and Darts	22.67 $\pm$ 3.13	18.22 $\pm$ 2.47	15.38 $\pm$ 3.78 [#]	12.63 $\pm$ 1.26 [#]
Solicitations	6.11 $\pm$ 1.399	3.56 $\pm$.82	3.38 $\pm$.532	4.13 $\pm$ 1.27
FLC	106.11 $\pm$ 11.11	101.22 $\pm$ 10.03	93.88 $\pm$ 8.46	91.88 $\pm$ 9.77
Lordosis Ratio	2.54 $\pm$.21	2.75 $\pm$.04	2.73 $\pm$.05	2.66 $\pm$.09
Hit Rate	.88 $\pm$.03	.86 $\pm$.03	.90 $\pm$.04	.86 $\pm$.05

Table 8

Food intake as a function of group and time after treatment (mean $\pm$ SEM).

Time	Treatment			
	Ad Libitum		16-Hour Food Deprivation	
	Ghrelin	Saline	Antagonist	Saline
1-Hour	1.85 $\pm$.49	2.37 $\pm$.61	5.11 $\pm$.39 [#]	4.81 $\pm$.34 [#]
2-Hours	4.42 $\pm$.96	4.21 $\pm$.48	5.76 $\pm$.92 [#]	8.24 $\pm$.79 [#]
24-Hours	29.74 $\pm$ 1.19	29.77 $\pm$ 1.35	34.10 $\pm$ 1.91 [#]	38.99 $\pm$.81 [#]

[#] $p < .05$ from ad libitum groups

Figure 1: The molecular format of ghrelin. Only in it's acylated form, can ghrelin bind to its receptor, growth hormone secretagogue receptor (GHSR).

Figure 2: Anticipatory male level changes. A) No significant differences in level changes during the first 5-minute anticipatory interval, $F_{(1,18)} = 3.208, p = .09, \eta_p^2 = .151$. B) Ghrelin decreases anticipatory behavior over a 10-minute interval as compared to saline, $F_{(1,18)} = 23.965, p = .001, \eta_p^2 = .571$.

Figure 3: No significant changes in the number of mounts (A) or intromission (B) or ejaculations (C) between males receiving intra-mPOA ghrelin or saline, $F_{(1,18)} = 1.10, p = .345, \eta_p^2 = .064$, $F_{(1,18)} = .565, p = .573, \eta_p^2 = .032$, and $F_{(1,18)} = 1.659, p = .215, \eta_p^2 = .089$ respectively.

Figure 4: No significant differences in the latency to mount (A) or intromit,(B); $F_{(1,18)} = .303$, $p = .539$, $\eta_p^2 = .024$ and $F_{(1,18)} = .105$, $p = .750$, $\eta_p^2 = .006$, respectively. Males receiving intra-mPOA ghrelin microinjections displayed a shorter latency to ejaculate when compared to saline , $F_{(1,18)} = 5.617$, $p = .029$, $\eta_p^2 = .238$.

* $p < .05$ from ghrelin.

Figure 5: No significant differences of food intake were seen at 1-, 2-, and 24-hours: $F_{(1,18)} = .447, p = .512, \eta_p^2 = .024$, $F_{(1,18)} = 2.312, p = .146, \eta_p^2 = .114$ and 24-Hour $F_{(1,18)} = 1.975, p = .177, \eta_p^2 = .099$, respectively

Figure 6: Placements for the 33 males in Experiment 1. Nine out of the 33 males used did not recover from surgery or get through all of the 2 baseline tests and 3 experimental tests, and were thus sacrificed and removed from the data set. Furthermore, 5 animals were removed due to incorrect placements.

Figure 7. A) Ad libitum (AdLib) males displayed significantly more level changes during the first 5-minutes of the anticipatory period when compared to the food deprived (FD) group, $F_{(1,16)} = 12.642$, $p = .003$, $\eta_p^2 = .441$. B) Males on AL displayed significantly higher anticipatory behaviors, as measured by level changes during a 10-minute interval, when compared with FD males, $F_{(1,16)} = 24.904$, $p = .000$, $\eta_p^2 = .609$. Administration of an antagonist significantly reduced anticipatory behavior when compared to saline under a FD dietary schedule, $F_{(1,7)} = 7.519$, $p = .024$, $\eta_p^2 = .518$. * $p < .05$, ** $p < .01$

Figure 8: There were no significant differences in the number of mounts (A), intromissions (B) or ejaculations (C) between AD males and FD males, $F_{(1,15)} = .537, p = .479, \eta_p^2 = .034$, $F_{(1,15)} = 2.326, p = .148, \eta_p^2 = .134$, and $F_{(1,15)} = 1.954, p = .182, \eta_p^2 = .115$

Figure 9: No significant differences between the latency to mount, intromit or ejaculate $F_{(1,15)} = 3.242, p = .092, \eta_p^2 = .178, F_{(1,15)} = .332, p = .573, \eta_p^2 = .022$ and $F_{(1,15)} = 1.176, p = .297, \eta_p^2 = .077$, respectively.

Figure 10: Males on FD ate significantly more than AL animals at 1-, 2-, and 24- Hours post testing: $F_{(1,15)} = 34.786, p = .000, \eta_p^2 = .699$, $F_{(1,15)} = 15.093, p = .001, \eta_p^2 = .502$ and $F_{(1,15)} = 4.401, p = .053, \eta_p^2 = .227$ respectively. No significant differences within groups was seen at 1-, 2-, and 24- hours, $p > .05$ for all.

Figure 11: Placements for the 24 males in Experiment 2. Twenty-one out of the 24 males recovered from surgery, and survived all experimental sessions. Four animals were removed from the data set for incorrect placements.