Ghrelin, Cannabinoids, and Motivation: Increased Food Motivation on Progressive Ratio Paradigm Through MAGL/FAAH Inhibition in a Rat Model

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

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Abstract

Ghrelin is a stomach derived hormone that is popularly known for its orexigenic properties. Ghrelin acts on its receptor (the GHS-R) in the hypothalamus and ventral tegmental area (VTA) to increase both homeostatic and non-homeostatic feeding, respectively. Endogenous cannabinoid system also produces feeding effects via the cannabinoid receptor in these regions. While there is evidence of ghrelin requiring the endogenous cannabinoid system to produce its effects in the hypothalamus, how they interact within the VTA is not fully understood. It is hypothesized that ghrelin may influence this system by stimulating the release of endocannabinoids from dopamine cells through its action on the GHS-R, via increased synthesis and accumulation of membrane bound cannabinoid enzymes. To investigate this, we conducted two experiments. In experiment 1, we used a progressive ratio paradigm to examine the behavioural effects of blocking cannabinoid catabolism on ghrelin induced motivated feeding behaviours by administering an intra-VTA infusion of MAGL and FAAH inhibitors (MJN110/PF-00457845), followed by a threshold dose of acylated ghrelin. In experiment 2, we examined the protein level of cannabinoid related enzymes in the VTA following an injection of ghrelin using western blotting. Our results show a significant increase in motivated feeding behaviours in rats that received both the MAGL/FAAH inhibitor cocktail and ghrelin via increased lever pressing. Interestingly, western blot analyses showed an increase in the cannabinoid degrading enzyme MAGL and a decrease in the cannabinoid synthesizing enzyme DAGL-α. Together, our results suggest ghrelin and the endogenous cannabinoid system may interact within the VTA to produce feeding behaviours.
Acknowledgments

Firstly, I would like to thank my supervisor, Dr. Alfonso Abizaid, for taking a chance on me as an undergraduate, and for believing in my potential as a graduate student. You have taught me many lessons that go beyond the lab bench that I will keep with me for a lifetime. To my fellow lab members: thank you for being a rock, a smile and a helping hand. Long days and weekends in the vivarium were always made better with a friend. To my mother: your resilience and unconditional love is inspiring. Thank you for always understanding my hectic schedule, and for being at my door with a home cooked meal at a moment’s notice. To my partner, Patrick: thank you for believing in me and encouraging me to pursue graduate school. Your love for me did not waver, even while I was glued to my laptop at the kitchen table in our 400 square foot apartment with a 50lb dog in a pandemic. Thank you for being my voice of reason through it all. Finally, thank you to my lovely dog Luna, for the unconditional love and daily reminder to take a break and go for a walk.

I dedicate this thesis to my father, my #1 supporter.
List of Abbreviations

α-MSH, alpha-melanocyte stimulating hormone; Δ-9 THC, Δ-9-tetrahydrocannabinol; 2-AG, 2-arachidonoylglycerol; AEA, anandamide; AgRP, agouti-related protein; ANOVA, analysis of variance; ARC, arcuate nucleus of the hypothalamus; CB-1, cannabinoid receptor type 1; CB-2, cannabinoid receptor type 2; CCK, cholecystokinin; DA, dopamine; DAG, diacylglycerol; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; FR, fixed ratio; GABA, γ-aminobutyric acid; GHS-R, growth hormone secretagogue receptor; GOAT, ghrelin-O-acyltransferase; GPR55, G protein coupled receptor 55; IP, intraperitoneal; MAGL, monoacylglycerol lipase; MC3R, melanocortin receptor 3; MC4R, melanocortin receptor 4; NAc, nucleus accumbens; NAPE, n-acyl phosphatidylethanolamine; NAPE-PLD, n-acyl phosphatidylethanolamine phospholipase D; NPY, neuropeptide Y; PFA, paraformaldehyde; PLC, phospholipase C; POMC, proopiomelanocortin; PR, progressive ratio; PYY, peptide YY; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TRP-V1, transient receptor potential cation channel subfamily V member 1; VTA, ventral tegmental area
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Introduction

Food intake is influenced by various factors. One factor is circulating signals from tissues in the periphery that provide feedback on the metabolic state of the organism and include leptin, insulin, cholecystokinin (CCK), peptide YY (PYY), and ghrelin (Abizaid & Horvath, 2008). Of these, leptin, insulin, CCK and PYY are satiety signals and are released in response to high blood glucose or food intake, and act in the brain to reduce food intake (Abizaid & Horvath, 2008). Conversely, ghrelin is the only peripheral orexigenic signal and is secreted primarily from the stomach (Date et al., 2000). Together, these signals maintain energy homeostasis through acting on neurons in the hypothalamus (Broberger, 2005). However, food intake is not solely controlled by orexigenic and satiety signals. It is also influenced by the incentive value of food, such that palatable foods are eaten even when the organism is satiated, resulting in an excess of energy intake over energy expenditure (Monteleone et al., 2012). Having highly palatable food readily available and accessible to the average consumer is a contributing factor to the rise in rates of obesity (Leigh & Morris, 2018). It is well established in the literature that the control of homeostatic feeding is mediated primarily in the hypothalamus, while non-homeostatic feeding is believed to be influenced by the activity of dopamine (DA) cells in the ventral tegmental area (VTA) (Abizaid & Horvath, 2008). Ghrelin receptor activity has been implicated to modulate food intake at both sites (Abizaid, 2009). Recently it has been suggested that, specifically in the VTA, ghrelin interacts with the endogenous cannabinoid system to accomplish this (Edwards, 2020).

Ghrelin, a growth hormone-releasing acylated peptide, was first isolated in 1999 by Kojima et al. This 28-amino acid peptide was originally discovered to stimulate growth hormone release from the anterior pituitary (Müller et al., 2015), but more notably, ghrelin has a major
effect on appetite and body fat accumulation (for review see Abizaid, 2009). In addition, ghrelin plays a role in many other physiological processes, including the stimulation of gastrointestinal motility (Arosio et al., 2003).

Ghrelin was identified as the endogenous ligand of the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999) which has two subtypes: the GHS-R1a subtype is a g-protein coupled seven transmembrane protein with wide central and peripheral distribution. Peripherally, GHS-R1a is expressed in the gut, pancreas, liver and adrenal glands. In the brain, the GHS-R1a is expressed in high levels in the hypothalamus, anterior pituitary, olfactory bulbs, dentate gyrus, substantia nigra, nucleus of the solitary tract, ventral tegmental area, and the raphe nuclei (Gnanapavan et al., 2002; Müller et al., 2015). Ghrelin exerts its biological effects through its actions on this receptor subtype. The second receptor subtype is the GHS-R1b, a truncated isoform of the GHS-R1a. Ghrelin does not bind to this receptor despite the wide tissue distribution of this receptor isoform (Gnanapavan et al., 2002), although a recent study suggests that it forms oligomers with the GHS-R1a and other g-coupled protein receptors as part of the mechanisms underlying ghrelin signaling (Abizaid & Hougland, 2020; Navarro et al., 2021).

Ghrelin binding to its receptor is dependent on a post translational modification on serine-3 of its peptide mediated by ghrelin-O-acyltransferase (GOAT) (Abizaid & Hougland, 2020). The conformational alteration of ghrelin into its active constituent acyl-ghrelin, occurs prior to secretion into the bloodstream (Bednarek et al., 2000). GOAT varies in its expression, mirroring ghrelin secretion through exhibiting peak levels during the fasted state (Kirchner et al., 2009). Acyl ghrelin is converted to des-acyl ghrelin through esterase-catalyzed diacylation of the serine octanoyl ester about 30 minutes after secretion (De Vriese et al., 2004). Des-acyl ghrelin is the most prevalent form of circulating ghrelin in humans, with only 10% of that ghrelin being
acylated and capable of stimulating the GHS-R (Patterson et al., 2005). Additionally, there is evidence of GOAT expression centrally within the hypothalamus potentially converting des-acyl ghrelin into acyl-ghrelin (Wellman & Abizaid, 2015). When rats were fed a high fat diet and received a knockdown of central GOAT expression via chronic intracerebroventricular infusion of a GOAT antisense-morpholino, a reduction in weight gain and caloric efficiency was observed (Wellman & Abizaid, 2015). Ultimately, the literature describing the role of des-acyl ghrelin in feeding behaviours is unclear (Asakawa et al., 2005; Neary et al., 2006).

Energy homeostasis can be defined as the balance between energy intake and energy expenditure. The central nervous system integrates neuronal, metabolic and endocrine signals to assess energy levels, and produces behavioral, physiological and cellular responses aimed to attain energy balance (Spiegelman & Flier, 2001). To this end, a number of hormones are secreted by various tissues including adipose tissues, liver, reproductive organs, muscles the gastrointestinal tract (Havel, 2001). These signals target the hypothalamus and the brain stem, to increase or decrease food intake as required (Broberger, 2005). Ghrelin, corticosterone, leptin, PYY(3-36), insulin and cholecystokinin are among some of the hormones that regulate feeding and energy balance (Abizaid & Horvath, 2008).

Of all of these hormones, ghrelin is the only gastrointestinal peptide that stimulates food intake (Broberger, 2005). Ghrelin is secreted during caloric restriction and circulates in the blood at high levels in fasted rats (Cummings et al., 2001). Ghrelin increases in anticipation of a meal, with its secretion and expression decreasing soon after the initiation of food intake (Cummings & Shannon, 2003). Both peripheral and central administration of ghrelin causes increased food intake and weight gain by reducing fat utilization in rats and mice (Shuto et al., 2002). It is generally accepted that a major route through which ghrelin has its effects is via the activation of
the GHS-R1a on neurons in the arcuate nucleus. Moreover, if animals or humans injected with ghrelin are given a choice, they increase their consumption of palatable foods (Abizaid, 2009; Schmid et al., 2005), suggesting that ghrelin not only increases food intake but also enhances the preference for palatable foods (Vengeliene, 2013). In this sense, feeding behaviour can occur in the absence of negative energy states, and driven by the incentive value (palatability) of different foods (Skibicka et al., 2013).

**Ghrelin and Homeostatic Feeding**

The role of the hypothalamus in regulating food intake and energy homeostasis was established in 1940 by Hetherington and Ranson. Bilateral electrolytic lesions to the ventromedial hypothalamic area of rats showed a large increase in body weight (Hetherington & Ranson, 1940). Later it was found that specifically lesioning the lateral hypothalamus adjacent to the ventromedial nucleus caused a loss of feeding (Anand & Brobeck, 1951). Thus, the hypothalamus became known for its role in food intake, with the lateral area impacting feeding, and the ventromedial area impacting satiety. The hypothalamus is an important brain region in this chain of action, as it is the center of central and peripheral information integration (Broberger, 2005). The neural pathway from the hindbrain to the hypothalamus is important for the communication of feeding information from the periphery, and energy homeostasis as a whole (Utoyama et al., 2016). When this pathway is severed, rats show a significant increase in their food intake, body weight and leptin levels, as well as an insulin resistance (Utoyama et al., 2016).

The lesions caused by Hethering and Ranson (1940) included a brain region now known as the arcuate nucleus of the hypothalamus (ARC). It is interesting to note that the ARC is a
circumventricular brain region where the blood-brain barrier is more permeable, thus allowing for enhanced access to signals from the periphery (Abizaid, 2009). The ARC contains some of the highest concentrations of GHS-R in the brain (Abizaid, 2009). This region also contains two distinct populations of neurons that have opposing effects on feeding and energy expenditure: agouti-related protein (AgRP) / neuropeptide Y (NPY) expressing neurons which promote feeding and reduce energy expenditure, and proopiomelanocortin (POMC) expressing neurons which suppress feeding (Hahn et al., 1998). Interestingly, the GHS-R1a is colocalized in AgRP/NPY expressing neurons but not in POMC neurons, explaining ghrelin’s mode of action (Takahashi & Cone, 2005; Willesen et al., 1999).

The peptide AgRP is the most potent orexigenic and is only synthesized in the ARC (Abizaid & Horvath, 2008). AgRP is co-expressed with NPY in this brain region, and together they increase appetite, food intake and lipogenesis, while decreasing metabolic rate and energy expenditure (Leibowitz et al., 2005). When in a fasted state, hypothalamic AgRP/NPY neurons stimulate food intake by increasing NPY and while decreasing the secretion of alpha-melanocyte stimulating hormone (α-MSH), a cleaved peptide derived from the POMC pre-pro peptide and one that decrease appetite (Hahn et al., 1998). An increase in AgRP neuron firing can be seen in brain slices of fasted mice (Takahashi & Cone, 2005). Furthermore, NPY expression increases before scheduled food intake to aid meal initiation (Kalra et al., 1991). AgRP also increases appetite but it does so in part through competitive binding to melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), where AgRP acts as an endogenous antagonist and α-MSH acts as an endogenous agonist (Takahashi & Cone, 2005). Nevertheless, optogenetic activation of AgRP neurons results in increased feeding despite inactivation of these receptors suggesting that AgRP does not require melanocortin pathway suppression via POMC to engage feeding.
circuits (Aponte et al., 2011). It is likely that optogenetic activation of AgRP neurons results in the release of GABA, another neurotransmitter released by NPY/AgRP neurons, to inhibit the activity of nearby POMC neurons (Cowley et al., 2001). Ghrelin stimulates the release of AgRP/NPY from the ARC, modulating the activity of these cells through the synaptic modeling of neuronal inputs (Hahn et al., 1998). AgRP and NPY are a crucial aspect of ghrelin’s actions on feeding behaviours, as mice that are lacking in both AgRP and NPY neurons do not increase food intake when administered peripheral ghrelin (Chen et al., 2004).

**Ghrelin and Non-homeostatic Feeding**

The mesocorticolimbic dopaminergic system modulates emotion-related behaviours and is the area of action of drugs with abuse potential (Arias-Carrión et al., 2010). Central to this system are dopamine (DA) producing cells in the ventral tegmental area (VTA) of the midbrain, which project axons to various limbic structures, including the nucleus accumbens (NAc), amygdala, and prefrontal cortex (Volkow et al., 2017). Midbrain DA neurons are critical in a variety of functions, such as creating associations between rewarding stimuli, motivation, working memory, emotion regulation and voluntary movement. Proper function and development of DA neurons is therefore vital, with dysfunctions being associated with a variety of ailments, including mental illnesses and drug addiction (Di Chiara, 2002). Midbrain DA neurons account for 75% of all dopaminergic neurons in the brain (Wallén & Perlmann, 2003). Neurons that produce γ-aminobutyric acid (GABA) are also found in the VTA, and these are thought to tonically inhibit the activity of DA producing cells (Van Bockstaele & Pickel, 1995).

The projections of DA neurons in the VTA are extensive and diverse in function (Bjorklund & Dunnett, 2007). The VTA receives input signals from a many regions such as the
hypothalamus, nucleus accumbens, amygdala, hypothalamus and medial prefrontal cortex (Beier et al., 2015). Within the VTA, DA is released in response to or in anticipation of rewards, as well as in response to stress (Schultz, 1998). These rewards include food rewards, as mice that cannot synthesize striatal DA die of starvation unless they are supplemented with the DA precursor L-DOPA (Cannon et al., 2004).

Approximately 60% of VTA dopaminergic neurons and 30% of GABAergic neurons express GHS-Rs, with intra-VTA administration of ghrelin increasing food intake of both regular food and that of palatable foods (Abizaid et al., 2006; Egecioglu et al., 2010; King et al., 2011; Naleid et al., 2005; St-Onge et al., 2016). Food restricted rats that receive an infusion of ghrelin into the VTA will work harder to receive a food reward compared to controls demonstrating increased motivation food behaviours, whereas a GHS-R antagonist reduced the number of bar presses produced to obtain food (King et al., 2011). Intra-VTA continuous administration of ghrelin also increases cue-induced reinstatement of palatable food rewards in satiated rats (St-Onge et al., 2016). These behavioural effects are also associated with increases in DA release into the nucleus accumbens in animals receiving ghrelin infusions in the VTA (Abizaid et al., 2006; Jerlhag et al., 2007).

At the cellular level, VTA DA neurons express the GHS-R and respond to ghrelin by increasing their frequency of action potentials (Abizaid, et al, 2006). The increases in the activity of VTA DA cells in response to ghrelin is due to a combination of pre and postsynaptic mechanisms that require a more in-depth investigation. But, at the presynaptic level, these involve plastic changes that increase presynaptic excitatory inputs (Abizaid et al., 2006). These
ghrelin induced changes also increase the likelihood of firing of dopamine neurons (Abizaid, 2009).

Endogenous Cannabinoids and Homeostatic Feeding

Like ghrelin, endogenous and exogenous cannabinoids produce an increase in appetite. Δ-9-tetrahydrocannabinol (Δ-9 THC), a compound derived from *Cannabis Sativa* was first isolated in 1964, and is now known for its psychoactive and orexigenic effects (Agurell et al., 1986; Gaoni & Mechoulam, 1964). Cannabinoids like Δ-9 THC mimic the effects of endogenous cannabinoids (endocannabinoids) by binding to endocannabinoid receptors located throughout the body (Svíženská et al., 2008). The actions of the endocannabinoid system are implicated in many functional mechanisms, such as cognitive functioning, and the regulation emotional and motivational states (Mechoulam & Parker, 2013). Cannabinoids affect neuronal functioning through inhibition of neurotransmitter release from presynaptic terminals (Gifford et al., 1997; Schlicker et al., 1997; Shen et al., 1996).

The cannabinoid receptor type 1 (CB-1) receptor is the most abundant g-coupled protein receptor in the central nervous system (Tsou et al., 1998a). CB-1 receptor activation mediates all physiological actions of endocannabinoids in the brain (Matsuda & Young, 1990). Specifically, this receptor is found in abundance within the prefrontal cortex, nucleus accumbens, amygdala, paraventricular nucleus of the hypothalamus, hippocampus, and basal ganglia (Howlett et al., 1990; Mailleux & Vanderhaeghen, 1992). Within the ventral tegmental area CB-1 receptors exist in low intensity, though many CB-1 receptors are found on axon terminals (Edwards & Abizaid, 2016; Svíženská et al., 2008). The cannabinoid receptor type 2 (CB-2) receptor was first considered to be found exclusively in the periphery (Munro et al., 1993), but it is now known
that they are present in the brainstem, frontal cortex, hippocampus, amygdala and VTA (Onaivi et al., 2006; Van Sickle et al., 2005). Additionally, cannabinoids bind to additional receptors including the transient receptor potential cation channel subfamily V member 1 (TRP-V1) and the orphan G protein coupled receptor (GPR55), both also expressed in the VTA (Baker et al., 2006; Zygmunt et al., 2013).

Anandamide (AEA) was the first endogenous cannabinoid identified (Devane et al., 1992). Synthesized in the postsynaptic neuron, anandamide is a derivative of polyunsaturated fatty acids and has a high affinity for CB-1 receptors, though it binds to both receptor subtypes (Felder et al., 1995). The half-life of anandamide is very short, being hydrolysed by fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine (Deutsch & Chin, 1993; Fezza et al., 2014). FAAH is a key regulator of anandamide signaling, as mice lacking the enzyme FAAH show an increase in anandamide levels, as well as an increase in CB-1 dependent behaviours (Cravatt et al., 2001) (see figure 1).

The second endogenous cannabinoid isolated was 2-arachidonoylgllycerol (2-AG) in 1995 (Mechoula et al., 1995; Sugiura et al., 1995). 2-AG is recognized as the primary endogenous cannabinoid in the brain because it exists in higher concentrations than anandamide. Although 2-AG has a lower affinity to the CB-1 receptor than anandamide, it is a full agonist at the CB-1 receptor (Childers & Breivogel, 1998). Activity of 2-AG is implicated in immune function, hippocampal long-term potentiation, neuroprotection, and inflammatory responses (Svíženská et al., 2008). Activation of cells that increase diacylglycerol (DAG) also activates the activity of diacylglycerol lipases (DAGL), DAGL-α and DAGL-β, which hydrolyze DAG and convert it into 2-AG (Di Marzo et al., 2015). In the brain, DAGL-α is the primary enzyme converting DAG into 2-AG (Di Marzo et al., 2015). Once released, 2-AG is hydrolyzed into arachidonic
acid and glycerol through the enzyme monoacylglycerol lipase (MAGL), found mainly in presynaptic terminals (Dinh et al., 2002). Manipulating MAGL through inhibition is an effective way of increasing 2-AG levels in the brain (Sticht et al., 2018).

Endogenous cannabinoids 2-AG and AEA act to regulate feeding within the hypothalamus and VTA through activation of the CB-1 receptor (Tsou et al., 1998b). Similarly to ghrelin, endogenous cannabinoid levels fluctuate throughout the day (Kirkham et al., 2002). After fasting, 2-AG levels in the hypothalamus of rats are increased, with these levels decreasing after the initiation of feeding (Kirkham et al., 2002). Furthermore, peripheral administration of CB-1 agonists or endogenous cannabinoids produces an increase in food intake, an effect that is not seen with administering a CB-1 receptor antagonist rimonabant, or in a CB-1 receptor knockout animal model (Gómez et al., 2002; Kirkham et al., 2002).
Figure 1. The action of ghrelin and endogenous cannabinoids at the synapse. Ghrelin produces its effects through activation of PLC signaling, which increases intracellular calcium via DAG (pathway illustrated in blue). Metabotropic glutamate receptors on the postsynaptic neuron stimulate PLC, beginning a signaling cascade to promote endogenous cannabinoid release (pathway illustrated in orange). Illustration data adapted from: Castillo et al., 2012; Edwards & Abizaid, 2016; García et al., 2001; Kojima et al., 1999; Malagón et al., 2003; Yin et al., 2014. AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB-1, cannabinoid receptor type 1; DAG, diacylglycerol; DAGL-α, diacylglycerol lipase-alpha; FAAH, fatty acid amide hydrolase; GHS-R, growth hormone secretagogue receptor; MAGL, monoacylglycerol lipase; NAPE, n-acyl phosphatidylethanolamine; NAPE-PLD, n-acyl phosphatidylethanolamine phospholipase D; PLC, phospholipase C. Created with BioRender.com.
**Endogenous Cannabinoids and Non-homeostatic Feeding**

Endogenous cannabinoids and CB-1 receptors are found within the VTA and nucleus accumbens shell, both regions involved in reward and motivation (Di Marzo et al., 2009). Administering rimonabant reduces the increase of extra-cellular DA in the NAc that is typically induced by palatable foods and therefore decreases the reinforcement aspect of high calorie food rewards, supporting the role of endocannabinoids in food motivated behaviours (Maccioni et al., 2008; Melis et al., 2007). Galette et al. (1999) found that CB-1 receptor agonists increase the motivation to obtain palatable food and drink rewards in rodents as reflected in an increase in breakpoint on progressive ratio task (Gallate et al., 1999).

DA release to the nucleus accumbens shell is critical for the addictive and rewarding properties of palatable foods and in the compulsive aspects of disorders like binge eating disorders, and drug abuse (Avena, 2007; Volkow et al., 2004). Genetic ablation of the CB-1 receptor or intraperitoneal injections of the CB-1 receptor antagonist SR141716A leads to a decrease in alcohol, nicotine or morphine self-administration, in association with decreased DA release in the nucleus accumbens shell (Cippitelli et al., 2005; Cohen et al., 2002; Maldonado et al., 2006; Vinklerová et al., 2002). Similarly, VTA administration of a CB-1 receptor agonist increases DA release in the accumbens, which is significantly reduced by GHS-R-1a antagonist administration (Charalambous et al., 2020). Further, microinjections of Δ-9 THC into the posterior VTA induced drug self-administration and established conditioned placed preference, whereas infusion of the CB-1 receptor antagonist rimonabant decreased drug self-administration (Zangen et al., 2006). Given that the posterior VTA contains GABA and glutamate neurons that tonically inhibit DA release, one could propose that cannabinoids act as retrograde modulators of glutamatergic and GABAergic VTA DA neuron afferents (Katona et al., 2001).
**Ghrelin and the Endogenous Cannabinoid System**

Within the brain, there is a large degree of overlap in the localization of GHS-R and cannabinoid receptors (Abizaid, 2009; Morton et al., 2006). Interactions between the ghrelin and the cannabinoid systems in the control of food intake have been reported in the hypothalamus. For example, infusing ghrelin into the PVN stimulates food intake, and these effects are attenuated by administration of the CB-1 receptor antagonist rimonabant (Tucci et al., 2004). Additionally, removing the CB-1 receptor genetically or inhibiting 2-AG production through inhibition DAG lipase produces the same results (Kola et al., 2008). The mechanism of interaction between cannabinoids and ghrelin appears to be complex and to involve non-canonical cannabinoid actions. It seems that both ghrelin and cannabinoids increase the activity of the AMP-activated protein kinase (AMPK) activity in the hypothalamus, to modulate energy substrate utilization at the level of the mitochondria, and the absence of GHSR or CB-1 prevent the activation of this pathway by cannabinoids or ghrelin respectively (Kola et al., 2005).

Within the VTA, the GHS-R1a is expressed in DA cells and potentially in pre-synaptic inputs to DA neurons (Abizaid et al., 2006). Similarly, cannabinoid receptors are located in afferent synapses to VTA neurons as well as in DA neurons themselves (Di Marzo et al., 2009). Two recent papers demonstrated that VTA CB-1 receptors mediate the feeding effects of ghrelin delivered into the VTA in mice and rats (Edwards, 2020; Kalafateli et al., 2018). In these papers, intra-VTA infusions of ghrelin increased food motivation and this effect was attenuated when animals were pre-treated with rimonabant. Interestingly, it is likely that the mechanisms of interaction between the GHSR and the CB-1 receptor in the modulation of activity of DA neurons is more canonical (Edwards, 2020). Given that both cannabinoids and ghrelin stimulate feeding through the activation of DA cells, a hypothesis can be drawn that the ghrelin and
endocannabinoid system interact to modulate food motivation via the stimulation of the DA system.

**Purpose of Thesis**

Though both CB-1 and GHS-R1a receptors are found within the VTA, and there is substantial evidence that they interact in the control of food intake, the precise mechanism underlying this interaction is not defined. Our lab has found that GHS-R knockout rats have a significant decrease in VTA 2-AG concentrations, as well as a significant decrease in gene expression of important transcripts for cannabinoid enzymes DAGL-α and FAAH in the VTA, suggesting an interaction between ghrelin and the endogenous cannabinoid system (Edwards, 2020). Furthermore, ghrelin’s ability to induce motivated feeding behaviour is decreased when CB-1 receptors are antagonized by rimonabant (Edwards, 2020).

In this thesis we hypothesize that within the VTA, ghrelin stimulates endogenous release of 2-AG to produce an increase in motivated feeding behaviours. While our lab has demonstrated that ghrelin increases 2-AG content in the VTA, we do not know if this increase is critical for enhancing food motivation. One way to investigate if 2-AG production following ghrelin administration results in increased food motivation is to deliver ghrelin at a dose that is not effective in producing behavioral appetitive responses, and determine if blocking enzymes that degrade 2-AG (i.e. MAGL and FAAH) within the VTA will enhance the effects of this low dose of ghrelin to increase food motivation as measured with the progressive ratio paradigm. Furthermore, we will examine if ghrelin administration alters protein levels of 2-AG regulating enzymes including MAGL, FAAH and DAGL-α. We predict that blocking 2-AG degradation will enhance the effects of ghrelin on food motivation as reflected in increased effort to obtain
food on an operant progressive ratio task. Furthermore, we predict that ghrelin administration will also alter protein content of enzymes associated with the generation and degradation of 2-AG.

**Methods**

*Experiment 1: MAGL + FAAH inhibition facilitate the effects of ghrelin on motivated feeding behaviours within the VTA*

**Animals**

48 male Long Evans rats (Charles River, St. Constant, Canada), weighing 276-300 g (7-9 weeks), were used for the operant conditioning paradigm. Upon arrival, rats were single housed and habituated to the vivarium for one week, with temperature (22°C) and humidity (45-55%) controlled. They were housed in plexiglass cages (48 cm x 26 cm x 20 cm) and maintained on a 12-hour light-to-dark cycle (lights on from 08:00 to 20:00). The rats had free access to water and standard chow (Harlan Diets; 3.3 kcal. g⁻¹; 4% fat; 48% carbohydrates; 14.3% protein), unless otherwise specified. All procedures described here were approved by the Carleton University Animal Care Committee and followed the guidelines laid out by the Canadian Council on Animal Care.

**Operant Conditioning Procedure**

The operant conditioning procedure was conducted in 6 identical operant conditioning chambers (12" W x 10" D x 12" H; Coulbourn Instruments), situated inside isolation cubicles (30" W x 17.75" D x 18.5" H; Coulbourn Instruments). Each chamber was equipped with a steel
grid floor and two stationary levers, situated 3/4” above the floor. The left lever was food-paired; presses on this lever delivered a food pellet through a nose hole in between the two levers. The right lever was inactive; presses on this lever had no effect. Throughout the experiment, the house light remained on. Lever presses, nose pokes and locomotor activity were recorded using Graphic State software (Coulbourn Instruments).

The full experimental timeline is outlined in figure 2. One week of baseline food intake and body weight measurements were taken for each animal. Rats were then fasted to 90% of their baseline body weight to facilitate learning. First, rats were trained to bar press on a fixed ratio 1 (FR-1) schedule. This means that for each press on the active lever, they received one food pellet reward. The active lever was primed with five food pellets to initiate pressing. Each training session occurred once daily for 30 minutes. Once the rats reliably responding on this schedule, they began an FR-3 schedule, where three active lever presses were needed to obtain one food reward. Once the animals reliably responded under this schedule, they were moved to an FR-5 schedule. Reliably responding to a lever pressing schedule is defined as less than 20% variance between active lever presses within the past three consecutive training days. Once animals were reliably responding to the FR-5 schedule, rats were returned to ad libitum food access for three days before surgery. On average, it took one animal 20 days to complete the entire training phase.

After recovery from surgery, rats were again food restricted again for 7 days and then retrained on FR-5 for four days. This was done to ensure the surgical procedure did not alter the previously established response rate. Rats were assigned to one of four treatment groups. When assigning animals to experimental groups, the average number of active lever presses during the four-day FR-5 retraining was calculated for each animal. Each experimental group was assigned
an equal number of ‘high pressing’ animals and ‘low pressing’ animals to remove biases and ensure counterbalancing (supplemental table 1).

The next day, rats were subjected to a mock test day on which they were exposed to the same intracranial infusion procedure as on the test day, but without receiving any drugs. The mock infusions serve to get the animals acclimated to the handling and sound of the infusion pump. On this day the rats were also place in the operant chamber and tested on the progressive ratio (PR) reinforcement schedule. Animals were then given ad libitum food access for three days before the progressive ratio test day. Each progressive ratio test was 120 minutes. After the final test, a 24-hour food measurement was taken.

The PR schedule was developed to assess the efficacy of a stimulus at enhancing motivated behaviours (Richardson & Roberts, 1996). On the PR schedule, the number of lever presses needed to obtain a food reward increases exponentially (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178 etc.), according to the equation: Response ratio (rounded to nearest integer) = \[5e^{(\text{number of rewards obtained x 0.2})} - 5\] (Richardson & Roberts, 1996). A break point was reached when the animal received the next food reward within 30 minutes of receiving the previous food reward. If the animal did not reach a new break point within 30 minutes, the recording ended, and the animals’ final break point reached was recorded.
Figure 2. Average time (days) of the operant conditioning experiment timeline per animal. FR, fixed ratio; PR, progressive ratio.
**Surgery Procedure**

Rats were anesthetized with isoflurane (5% induction, 3% maintenance), and given an injection of slow release Metacam (2 mg/kg, subcutaneous) to relieve pain during recovery. After being placed in the stereotaxic apparatus (Kopf Instruments, Tujunga, CA), an incision was made, the skull cleaned, and a guide cannula (22 gauge; P Technologies) was inserted 1mm above the VTA. The following coordinates were used from Bregma: anterior-posterior (AP), -5.6 mm; medial-lateral (ML), +2.0 mm; dorsal-ventral (DV), -7.6 mm, angled towards the midline (10°) to avoid the aqueduct. The guide cannula was anchored to the skull using optical screws and dental cement. A dummy cannula (P Technologies) was then screwed into the guide cannula for the length of the experiment to prevent occlusions. At the end of surgery, the animals were placed in new clean cages on a heating pad (36°C) and monitored for two hours for surgical complications. Animals were given 7 days for post-operative recovery with ad libitum food and water access before continuing the experiment.

**Intracranial Infusions**

Rats were assigned to one of four treatment groups: DMSO + saline, DMSO + ghrelin, MAGL/FAAH inhibitor cocktail + saline, and MAGL/FAAH inhibitor cocktail + ghrelin. A micro infusion syringe (Braintree Scientific, Inc.) connected to 5 μl glass syringes and polyethylene-50 tubing was used for all infusions. The internal cannula (26 gauge; P Technologies) extended 1mm below the guide cannula. The first infusion of MJN110 (MAGL inhibitor, Cayman Chemical) was prepared at a concentration of 10 μg/μL and PF-04457845 (FAAH inhibitor, Tocris Bioscience) was prepared at 6 μg/μL. Doses were calculated based on previous research on the ability of MJN110 and PF-04457845 to sufficiently increase cortical 2-
AG and AEA (Parker et al., 2015; Sticht et al., 2018). Together, they were administered in a vehicle solution with a 1:10 ratio of dimethyl sulfoxide (DMSO) : saline at a final volume of 0.5 μL into the VTA. For the second infusion, acylated ghrelin (Tocris Bioscience) was prepared at a concentration of 1 μg/μL, dissolved in sterile saline, and administered at a final volume of 0.5 μL into the VTA. This dose is based on previously literature, indicating that administering less than 1 μg of ghrelin induces minimal behaviour outputs, with 1 μg of ghrelin previously shown to induce a feeding response (Abizaid et al., 2006; Naleid et al., 2005; Skibicka et al., 2011). The infusion lasted one minute followed by an additional minute to allow for full dispersion of the solution into the tissue.

Unilateral infusions of either the MAGL/FAAH inhibitor cocktail or vehicle (DMSO) were administered over 2 minutes; the internal cannula being left in place for an additional 30 seconds to ensure diffusion. The animals were then put back in their home cages. After 30 minutes, the second infusion was given of either ghrelin or saline over a 2-minute infusion time, with the internal cannula being left for an additional 30 seconds. Animals were then directly placed in the operant conditioning chamber for the progressive ratio paradigm.

**Cannula placement verification**

Animals were sacrificed via transcardial perfusion 24 hours after the end of the final test. Saline was circulated through the body to clear the blood using a perfusion pump (Masterflex) before circulating 4% paraformaldehyde (PFA). After fixation, the brains were extracted and stored in 4% PFA for 48 hours at 4ºC. The brains where then transferred to a 30% sucrose solution and stored at 4ºC until sliced. Using a CM1900 cryostat (Leica Biosystems Inc.), brains were coronally sectioned at 60 μm and counterstained using Cresyl Violet (Sigma-Aldrich). The
sections were then observed under a light microscope (Zeiss AxioPlan) using a 2.5x lens to assess cannula placements. Tissue photos were compared to a brain atlas (Paxinos and Watson, 1998) to verify the cannula delivered the treatments into the correct brain region.

**Statistical Analysis**

Data were analyzed by two-way analyses of variance (ANOVA), with the first infusion (i.e. drug or vehicle) as one independent factor and the second infusion (i.e. ghrelin or saline) as the second independent factor. Significant interaction effects between factors were analyzed using Tukey’s HSD post-hoc pairwise comparisons. Statistical analyses were conducted using SPSS (IBM) and GraphPad Prism was used to create figures depicting data collected (San Diego, CA). For clarity, results of post-hoc analyses are indicated by asterisks within the figures. Results are represented as the mean +/- the standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ denote the significance thresholds.

*Experiment 2: Effects of ghrelin treatment on protein expression of enzymes related to endogenous cannabinoid synthesis and degradation*

**Animals**

23 adult Long Evans rats (male = 13, female = 10) were group housed under standard laboratory conditions, at a temperature of 22° C and a humidity of 45-55%. Rats were housed in plexiglass cages (48 cm x 26 cm x 20 cm) and maintained on a 12-hour light-to-dark cycle (lights on from 08:00 to 20:00). The rats had free access to water and standard chow (Harlan Diets; 3.3 kcal. g⁻¹; 4% fat; 48% carbohydrates; 14.3% protein) throughout the experiment. All
procedures described here within were approved by the Carleton University Animal Care Committee and followed the guidelines laid out by the Canadian Council on Animal Care.

**Drug Application**

On the experimental day, food was removed from all cages and rats were given an intraperitoneal injection of either ghrelin (1 mg/kg; n = 13) or saline (n = 10). This dose reliably induces a feeding response based on previous literature (Abizaid et al., 2006; Naleid et al., 2005). After 30 minutes, their brains were extracted following rapid decapitation. Brains were snap frozen in ethanol cooled in dry ice and stored at -80°C until processing for western blot.

**Western Blotting**

Tissue punches were collected from the VTA to detect protein levels of MAGL, DAGL-\(\alpha\) and CB-1 receptor. They were then submerged in an extraction buffer (0.1% SDS, 1 mM Na ortho-vandate in 10 mM tris) homogenized by mixing with one tablet of protease inhibitor cocktail (Roche Diagnostics, Laval, QC), and sonicated (power level 15). The lysate was then centrifuged at 12,000 rpm for 10 minutes at 4°C, and supernatant assessed for protein concentration using Pierce’s BCA Protein Assay Kit (Thermo Fisher Scientific). Total protein was denatured by mixing the remaining protein sample with 5X loading buffer (25% glycerol, 25% \(\beta\)-mercaptoethanol, 15% SDS, 0.25% bromophenol blue) in a ratio of 4:1 and heated for 5 minutes at 105°C. Protein was then rapidly chilled on ice for 5 minutes before being stored at -20°C. Total protein samples (15 - 20 µg) were suspended in 5X loading buffer. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 7.5% - 12.5% separating gel, run at 140 V for one hour in running buffer. Using a Bio-Rad (Hercules, CA)
Mini-PROTEAN 3 Electrophoresis Module, the resulting protein was then transferred at 100 V for one hour in ice cold transfer buffer, onto PVDF membranes (MilliporeSigma, Burlington, MA). The membranes were dried overnight.

The membranes were rewet with methanol, then stained for total protein using 0.0005% Fast Green (Sigma). Membranes were rinsed using a wash solution (6.7% acetic acid in 30% methanol) before being blocked for 90 minutes in 0.5% fish gelatin in TBS buffer (10 mM Tris-base (pH 7.5), 150 mM sodium chloride). Membranes were then incubated with primary antibodies targeting MAGL (1:500; Ken Mackie), DAGL-α (1:500; Ken Mackie), and CB-1 (CB1-GP-Af530; 1:500; Frontier Institute) diluted in 0.5% fish gelatin in TBS-T buffer (10 mM Tris-base (pH 8.0), 150 mM sodium chloride, 0.1% Tween-20) for 2 hours. Excess antibody was discarded using four 5-minute washes of TBS-T. The membranes were incubated for 1 hour at room temperature in an agitated solution of IRDYE 800CW donkey anti-guinea pig (926-32411; LI-COR Biosciences) or goat anti-rabbit (926-32211; LI-COR Biosciences) IgG secondary antibody. Excess antibody was discarded using four 5-minute washes of TBS-T. Final washes of the membrane were done using two 5-minute washes of TBS followed by one 2-minute wash. Membranes were visualized while wet using the LI-COR Odyssey Fc system at 800CW for 6 minutes.

The molecular weights of proteins were estimated using Precision Plus Protein Standards Dual Color (Bio-Rad, Hercules, CA). Protein bands were quantified by densitometry using Empiria Studio software (V 1.3.0.83, LI-COR Biosciences, Lincoln, NB) and normalized against Fast Green total protein stain.
Statistical Analysis

Data were analyzed by two-way analyses of variance (ANOVA), with sex (i.e. male or female) as one independent factor, and injection (i.e. ghrelin or saline) as the second independent factor. Significant interaction effects between factors were analyzed using Tukey’s HSD post-hoc pairwise comparisons. Statistical analyses were conducted using SPSS (IBM) and GraphPad Prism was used to create figures depicting data collected (San Diego, CA). For clarity, results of post-hoc analyses are indicated by asterisks within the figures. Results were represented as the mean +/- the standard error of the mean (SEM). * p < 0.05, ** p < 0.01, and *** p < 0.001 denote the significance thresholds.

Results

Experiment 1: MAGL + FAAH inhibition within the VTA enhances the effects of a threshold dose of an intra VTA ghrelin infusion on motivated feeding behaviours

In total, 22 animals were removed from the study. Thirteen rats did not recover from surgery or were sacrificed because they met end point criteria. Nine additional rats were removed due to incorrect cannula placements (figure 3 and 4). The data from these rats were not included in the statistical analyses. Thus, a total of 26 rats were included in the final analyses, resulting in a group membership of: DMSO + saline (n = 6), DMSO + ghrelin (n = 8), inhibitor cocktail + saline (n = 6), inhibitor cocktail + ghrelin (n = 6).

As shown in figure 5A, an intra-VTA administration of ghrelin in the absence of the combined MAGL and FAAH inhibitor increased the breakpoint but this increase failed to reach statistical significance (figure 5A, main effect of ghrelin: $F_{(1, 22)} = 3.374, p = 0.080, \eta^2_p = 0.133$).
Similarly, while ghrelin infusions at this dose seemed to increase total active lever presses until breakpoint, this increase failed to meet significance criteria (figure 5B, no main effect of ghrelin: $F_{(1, 22)} = 3.697, p = 0.068, \eta_p^2 = 0.144$). Similarly, intra-VTA MAGL and FAAH inhibitor cocktail administration did not significantly increase breakpoint (no main effect of inhibitor cocktail: $F_{(1, 22)} = 0.338, p = 0.567, \eta_p^2 = 0.015$) or total active lever presses until breakpoint (no main effect of inhibitor cocktail: $F_{(1, 22)} = 1.409, p = 0.248, \eta_p^2 = 0.060$). However, there was a significant interaction between intra-VTA ghrelin and MAGL and FAAH inhibitor cocktail treatments for both breakpoint ($F_{(1, 22)} = 6.661, p = 0.017, \eta_p^2 = 0.232$) and total active lever presses until breakpoint ($F_{(1, 22)} = 6.774, p = 0.016, \eta_p^2 = 0.235$). Tukey’s HSD post-hoc pairwise comparisons revealed a significant increase in breakpoint reached between animals that received DMSO X ghrelin versus the inhibitor cocktail X ghrelin ($p = 0.019$). Similarly, the active lever presses until breakpoint were significantly increased between animals that received DMSO X ghrelin versus the inhibitor cocktail X ghrelin ($p = 0.016$).

Locomotor activity (figure 5C), inactive lever presses (figure 5D) and nose pokes (figure 5E) were recorded as control measures to ensure the groups were acting in a similar matter. As shown in these figures, no significant differences were observed between groups in locomotor activity (main effect of ghrelin: $F_{(1, 22)} = 3.813, p = 0.995, \eta_p^2 = 0.000$; main effect of inhibitor cocktail: $F_{(1, 22)} = 0.389, p = 0.540, \eta_p^2 = 0.017$; interaction: $F_{(1, 22)} = 0.381, p = 0.544, \eta_p^2 = 0.017$), inactive lever presses (main effect of ghrelin: $F_{(1, 22)} = 0.050, p = 0.825, \eta_p^2 = 0.002$; main effect of inhibitor cocktail: $F_{(1, 22)} = 0.288, p = 0.597, \eta_p^2 = 0.013$; interaction: $F_{(1, 22)} = 1.460, p = 0.240, \eta_p^2 = 0.62$), or nose pokes (main effect of ghrelin: $F_{(1, 22)} = 0.720, p = 0.405, \eta_p^2 = 0.032$; main effect of inhibitor cocktail: $F_{(1, 22)} = 0.072, p = 0.790, \eta_p^2 = 0.003$; interaction:}
\[ F_{(1, 22)} = 0.544, \ p = 0.469, \ \eta_p^2 = 0.024 \). Drug treatment administration did not preferentially influence overall excitability.
Figure 3: Image taken of representative cannula placements for the ventral tegmental area.
Figure 4: Summary of cannula placements for Experiment 1. The red circles represent placements falling outside the VTA and excluded from all analyses. The green dots represent animals included in all analyses. Cannula placements were mapped using *The Rat Brain in Stereotaxic Coordinates* (Paxinos and Watson, 1998).
Figure 5: MAGL + FAAH inhibition alongside ghrelin increases motivated feeding behaviours within the VTA. Progressive ratio test (A) breakpoints, (B) active lever presses until breakpoint, (C) locomotor activity, (D) inactive lever presses, and (E) nose pokes following corresponding pharmacological treatments. Rats were infused (0.5 μl per infusion) into the VTA with either DMSO or MAGL + FAAH inhibitor cocktail (5 μg and 3 μg respectively) 30 minutes prior to saline or ghrelin (0.5 μg). Circles within each bar represents individual data points. * p < 0.05.
Experiment 2: Effects of ghrelin treatment on protein expression of enzymes related to endogenous cannabinoid synthesis and degradation

MA
gL

Representative images of molecular targets have been provided in figure 6. A total of 23 rats were included in the final analyses, with the final groups being: male + saline (n = 6), male + ghrelin (n = 7), female + saline (n = 4), female + ghrelin (n = 6). Western blot analyses of MAGL protein levels (~ 34 kDa) in the VTA after saline or ghrelin treatment revealed a significant sex X ghrelin interaction (figure 7A, $F_{(1, 19)} = 5.469$, $p = 0.030$, $\eta^2_p = 0.223$). Tukey’s HSD post-hoc pairwise comparisons revealed females that received ghrelin had significantly higher expression of MAGL protein compared to all other groups (male X saline: $p = 0.029$, male X ghrelin: $p = 0.037$, female X saline: $p = 0.019$).

DA
G
L-α

A total of 22 rats were included in the final analyses, with the final groups being: male + saline (n = 6), male + ghrelin (n = 6), female + saline (n = 4), female + ghrelin (n = 6). Analyses of VTA protein levels of DAGL-α protein levels (~110 kDa) revealed that ghrelin administration decreased DAGL-α protein levels (figure 7B, significant main effect of ghrelin, $F_{(1, 18)} = 6.979$, $p = 0.017$, $\eta^2_p = 0.279$). There was no significant main effect of sex ($F_{(1, 18)} = 0.069$, $p = 0.796$, $\eta^2_p = 0.004$) or sex X ghrelin interaction ($F_{(1, 18)} = 0.025$, $p = 0.877$, $\eta^2_p = 0.001$).

CB
-1 receptor

A total of 23 rats were included in the final analyses, with the final groups being: male + saline (n = 6), male + ghrelin (n = 7), female + saline (n = 4), female + ghrelin (n = 6). Multiple
bands were detected by the antibody, but only the band that fell at the proper weight (~64 kDa) was quantified. Western blot analyses of CB-1 receptor protein levels revealed a significant sex X ghrelin interaction (figure 7C, $F_{(1, 19)} = 10.00$, $p = 0.005$, $\eta^2_p = 0.345$). Tukey’s HSD post-hoc pairwise comparisons revealed a significant decrease in CB-1 receptor protein expression in males given saline versus males given ghrelin ($p = 0.018$).

Since there was no main effect of sex, males and females were pooled together and analyzed. These analyses revealed a significant increase in MAGL protein expression after ghrelin administration (figure 8A, $t_{(21)} = 2.116$, $p = 0.047$, $\eta^2 = 0.176$). In contrast, ghrelin treatment resulted in a decrease in DAGL-α protein content (figure 8B, $t_{(20)} = 2.854$, $p = 0.0098$, $\eta^2 = 0.289$). Finally, analyses of pooled data from males and females failed to detect changes in CB-1 receptor protein content in response to ghrelin (figure 8C, $t_{(21)} = 1.359$, $p = 0.189$, $\eta^2 = 0.081$).
Figure 6: Representative images of molecular targets. Solid arrowheads indicate average molecular weight (in kiloDaltons).
Figure 7: Western blot analyses. (A) MAGL, (B) DAGL-α, and (C) CB-1 receptor relative optical intensity of protein expression compared against total protein stain. Circles within each bar represents individual data points. * p < 0.05.
Figure 8: Western blot analyses. (A) MAGL, (B) DAGL-α, and (C) CB-1 receptor relative optical intensity of protein expression in both sexes compared against total protein stain. Circles within each bar represent individual data points. Blue circles indicate male subjects, and red circles indicate female subjects. * $p < 0.05$, ** $p < 0.01$. 
General Discussion

The present results sought out to determine if the endogenous cannabinoid system and ghrelin interact within the VTA to increase motivated feeding behaviours. We hypothesized that ghrelin acts on VTA dopamine cells to increase the release of 2-AG, and that inhibiting the degradation of 2-AG would result in the enhancement of the effects of ghrelin. We also investigated the hypothesis that peripheral ghrelin can also alter the protein content of cannabinoid synthetizing and degrading enzymes, as well as the amount of CB-1 protein in the VTA.

In our first study we found that an infusion of a threshold dose of ghrelin did not increase motivated feeding behaviours. Similarly, blocking MAGL and FAAH to prevent the degradation of baseline amounts of cannabinoids at the synapse did not increase motivated behaviours. Infusions of the same dose of ghrelin in combination with MAGL and FAAH inhibitors significantly increased food motivation as measured by increased amount of bar presses rats were willing to perform to receive a pellet of food. It has been seen previously that infusing a CB-1 receptor antagonist (rimonabant) directly into the VTA alongside ghrelin attenuates the motivated feeding behaviours of ghrelin in a similar progressive ratio paradigm (Edwards, 2020). Our results together with the above-mentioned results suggest that ghrelin increases cannabinoid release from VTA DA cells, and this release magnifies the effects of ghrelin on stimulating behaviours associated with food motivation (Edwards & Abizaid, 2016).

Previous experiments demonstrate that the ghrelin signaling pathway influences endocannabinoid content within the VTA, as GHS-R knockout animals show deficits in 2-AG content in the VTA and alterations in mRNA expression for CB-1 receptor and for enzymes associated with synthesis and degradation of 2-AG and AEA (Edwards, 2020). We therefore
examined, in experiment two, the ability of ghrelin to alter the expression of cannabinoid related enzymes using western blotting. Interestingly, and in contrast to what we expected, an intraperitoneal injection of ghrelin resulted in significantly decreased DAGL-\(\alpha\) protein expression in the VTA. Given that the protein samples collected came from cytosolic fractions, we are observing global protein levels. Therefore, it would be more appropriate to extract synaptosomes in future experiments to increase precision of the observed protein expression. Alternatively, these data may reflect a compensatory mechanism whereby ghrelin first leads to an increase in 2-AG and then becomes a feedforward signal that downregulates DAGL-\(\alpha\) in an attempt to curb the overproduction and release of cannabinoids at the synapse.

The increase in MAGL protein expression may be because of a similar mechanism. If ghrelin increases VTA 2-AG similarly as it does in the hypothalamus (Kola et al., 2008), this increase in MAGL may be a regulatory response of the synapse attempting to decrease the robust concentration of 2-AG. Surprisingly, post hoc comparisons reveal the increase in MAGL protein expression were primarily driven by data from females. While the sex difference in endogenous cannabinoid related enzymes within the VTA remains unclear, it is known that females have higher MAGL activity in the amygdala (Krebs-Kraft et al., 2010).

**Experimental considerations**

It is important to note that this research was conducted to investigate the degree that endogenous cannabinoid activity in the VTA mediates ghrelin’s ability to affect motivated feeding behaviours using behavioural and molecular techniques. While successful, there are some experimental considerations to be made.
Although western blotting is a superior technique for specificity and sensitivity, it is a technique that can be subjective and labor intensive. While conducting experiment two, a large amount of the immunoblots conducted did not produce any output. For each enzyme of interest, many antibodies needed to be tested until one produced a reliable output. It is for this reason that other enzymes of interest (i.e. NAPE-PLD, 2-AG, AEA, DAG) could not be included in the analyses. Empiria Studio software (V 1.3.0.83, LI-COR Biosciences, Lincoln, NB) was used to interpret and quantify blots and minimizing the subjective error of manual scoring, though there is still room for subjectivity in visualizing the membranes within the LI-COR fluorescence imaging machine. Furthermore, due to facility restrictions, the subjects used in experiment two were limited to tissues that were already available in storage. Thus, the sample sizes of each groups were disproportional and overall low and the ability to collect samples at different time points following ghrelin injections was curtailed. Overall, we know that ghrelin may influence the expression of cannabinoid related enzymes, but that does not conclude if ghrelin is altering the biochemical activity of these enzymes. Conducting different enzyme activity assays in similar samples following ghrelin injections can shed light on these issues. Therefore, our results should be considered with caution.

It is also a possibility that within the VTA, ghrelin and endocannabinoids may interact with other neurons besides dopamine neurons. In fact, both GABA and glutamate neurons are found within the VTA, and influence feeding behaviours (Creed et al., 2014; Dobi et al., 2010). Increased activity of VTA GABA neurons may reduce motivated behaviours through inducing acute anhedonia, and suppressing nearby DA neuron excitability (van Zessen et al., 2012). Additionally, VTA glutamate neuron activation increases its tone onto dopamine neurons to promote motivated behaviours (Wang et al., 2015). Since the GHS-R is generally excitatory, it is
possible ghrelin induces motivated feeding behaviours via glutamate neurons in the VTA, though it is unknown if the GHS-R is expressed on these neurons (Edwards, 2020; Yin et al., 2014). Interestingly, both GABAergic and glutamatergic neurons in the VTA express the CB-1 receptor, further demonstrating the need to understand how ghrelin and cannabinoids potentiate each other (Han et al., 2017).

**Research extensions**

Our results demonstrate that raising endogenous cannabinoid tone within the VTA increased ghrelin’s ability to induce motivated feeding within the VTA. However, our data only sheds light on the degrading pathways of endocannabinoids via MAGL and FAAH manipulation. DAGL-α is found presynaptically and is the main synthesizing enzyme of 2-AG (Di Marzo et al., 2015). Manipulating DAGL-α activity in the VTA of ghrelin treated rats and then testing them for food motivation using the progressive ratio paradigm will further clarify how the endogenous cannabinoid system interacts with ghrelin within the VTA. Administering a DAGL-α antagonist would decrease the overall endocannabinoid tone through decreasing 2-AG, providing an inverse manipulation to what we have conducted in experiment one. Our progressive ratio paradigm could also be expanded to include a GHS-R knockout animal model. Previous research has established that a CB-1 receptor agonist does not induce feeding in GHS-R knockout animals as it does in wildtypes (Lim et al., 2013). Using a GHS-R knockout model alongside manipulations to the endocannabinoid system will clarify the interconnection of the two systems. Additionally, infusing increasing concentrations of ghrelin alongside the proposed cannabinoid manipulations will help to expand our current findings.
In experiment two, western blotting analyses were used to determine protein expression of various endocannabinoid related proteins in male or female rats given an intraperitoneal injection of ghrelin. Further experiments can be done looking at protein expression of rats given the same pharmacological manipulations as in the progressive ratio paradigm (i.e. MAGL + FAAH inhibitor cocktail) to better understand our results. Additionally, including female rats in future progressive ratio experiments will help uncover our sex results in experiment two.

Conclusion

Together, ghrelin signaling as well as the endocannabinoid system are both important in non-homeostatic feeding, energy balance regulation and motivated behaviours. Understanding how these two systems interact with VTA dopaminergic neurons will enhance the body of literature of food addictions, drugs of abuse and obesity. Furthermore, expanding the knowledge on how ghrelin works in the brain contributes to understanding of the brain-gut axis, and how it is implicated in the everyday human experience.
### Appendix: Supplementary table

<table>
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<tr>
<th>Group</th>
<th>Mean lever presses</th>
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<td>DMSO + Saline</td>
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<tr>
<td>DMSO + Ghrelin</td>
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<td>MAGL/FAAH Inhibitor + Ghrelin</td>
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*Table 1:* Treatment group counterbalancing. Average number of lever presses on post-surgery FR-5 training per animal were calculated and used to determine treatment group assignment. The aim was to make each treatment group mean as equal as possible to counterbalance the high-pressing animals and the low-pressing animals.
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