**Dinner for two: Digging into how ghrelin and endocannabinoid systems interact
in the ventral tegmental area to regulate non-homeostatic feeding**

by

Alexander Edwards

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Department of Neuroscience
Carleton University
Ottawa, Ontario, Canada

0.1 Abstract

Ghrelin, a hormone produced by the stomach during times of energy deficiency, potently enhances feeding by activating its receptor, the growth hormone secretagogue receptor (GHSR). In the brain, GHSRs are highly expressed within the hypothalamus (HYP) and ventral tegmental area (VTA), brain regions responsible for regulating hunger-driven and reward/motivated feeding, respectively. Interestingly, endogenous cannabinoids (i.e. endocannabinoids) induce similar feeding effects by stimulating cannabinoid receptors (CB-1Rs) in many of the same brain regions that highly express GHSRs, including the HYP and VTA. Recent evidence showed that ghrelin requires endocannabinoid signaling within the HYP to promote food intake. While independent GHSR or CB-1R activation within the VTA increases motivated feeding, it is not known whether the effects of ghrelin in the VTA are also dependent on the endocannabinoid system. This thesis aimed to determine if these systems interact within the VTA and to ascertain the extent and underlying mechanism by which CB-1R signaling may mediate the capacity of ghrelin to promote motivated feeding behaviours within this region. We determined that genetic disruption of GHSRs suppresses the gene expression of important endocannabinoid system proteins and lowers endocannabinoid levels within the VTA. Moreover, we demonstrated that pharmacological antagonism of VTA CB-1Rs attenuated the potent orexigenic and motivational capacity of intra-VTA ghrelin. Electrophysiological investigations indicated that CB-1R antagonism blocked the ability of ghrelin to promote excitatory drive to VTA dopamine neurons, but that ghrelin may independently and directly stimulate these neurons. Together, our data show that CB-1R signaling mediates ghrelin-induced motivated feeding behaviours in the VTA. Our findings suggest that, as in the HYP and VTA, ghrelin and endocannabinoid systems may interact in all brain regions where their receptors are jointly expressed.

0.2 Co-author

Alfonso Abizaid

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0.4 Dedication

I dedicate this thesis to my wife and soulmate Emilia. Emilia was my rock, my sounding board, and my voice of reason during tough times. Her constant support, reassurance, and love over the past five years kept me on track and motivated. I could not have accomplished this without her and truly owe all of this to her.

0.5 List of Abbreviations

α -MSH, α -melanocyte-stimulating hormone; **β -MSH**, beta-melanocyte-stimulating hormone; **Δ^9 -THC**, Δ^9 -tetrahydrocannabinol; **2-AG**, 2-arachidonoylglycerol; **AC**, adenylyl cyclase; **AEA**, anandamide; **AgRP**, agouti-related peptide; **AMP**, adenosine monophosphate; **AMPA**, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; **AMPK**, adenosine monophosphate kinase; **AP-5**, 2-amino-5-phosphonopentanoic acid; **ARC**, arcuate nucleus; **ATP**, adenosine triphosphate; **CaMKII**, calcium/calmodulin-dependent protein kinase II; **cAMP**, cyclic adenosine monophosphate; **CART**, cocaine and amphetamine regulated transcript neurons; **CB-1R**, cannabinoid receptor 1; **CB-2R**, cannabinoid receptor 2; **cdNA**, complementary DNA; **CNQX**, cyanquixaline; **CPP**, conditioned place preference; **DAG**, diacylglycerol; **DAGL- α** , diacylglycerol lipase- α ; **DMN**, dorsomedial nucleus; **DMSO**, dimethyl sulfoxide; **DREADD**, Designer-Receptors-Exclusively-Activated-by-Designer-Drugs; **EPSCs**, excitatory postsynaptic currents; **eEPSCs**, evoked excitatory postsynaptic currents; **eIPSCs**, evoked inhibitory postsynaptic currents; **FAAH**, fatty acid amide hydrolase; **fMRI**, functional magnetic resonance imaging; **FR**, fixed- ratio; **GABA**, gamma aminobutyric acid; **GHSR**, growth hormone secretagogue receptor; **GOAT**, ghrelin O-acyltransferase; **GPCR**, G protein coupled receptor; **GPR-55**, G protein coupled receptor-55; **HYP**, hypothalamus; **ICV**, intracerebroventricular; **IPSCs**, inhibitory postsynaptic currents; **IP₃**, inositol triphosphate; **KO**, knockout; **LHA**, lateral hypothalamic area; **MAPK**, mitogen activated protein kinase; **mRNAs**, messenger ribonucleic acids; **NA**, nucleus accumbens; **NAPE-PLD**, N-arachidonoyl phosphatidylethanolamine-phospholipase-D; **NMDA**, N-methyl-D-aspartate; **NPY**, neuropeptide Y; **PBS**, phosphate buffered saline; **PFC**, prefrontal cortex; **PKA**, protein kinase A; **PLC**, phospholipase C; **POMC**, proopiomelanocortin; **PR**, progressive ratio; **PVN**, paraventricular nucleus; **TRP-V1**, transient receptor potential cation channel subfamily V member 1; **UPC-2**, uncoupling protein 2; **VMN**, ventromedial nucleus; **VTA**, ventral tegmental area; **WT**, wild type

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0.8 Preface

Living organisms have evolved intricate mechanisms that allow them to secure, utilize, and store nutrients to ensure survival. In vertebrates, many of these processes include the development of a rich behavioural repertoire to obtain food, as well as complex processes that allow for the utilization of nutrients and the storage of extra resources for future use, all aimed at maintaining homeostasis (Magni et al., 2009). As such, vertebrates initiate feeding behaviours in response to negative energy balance states (commonly termed homeostatic feeding behaviours), but also display feeding behaviours in anticipation of potential energy shortages (often referred to as non-homeostatic feeding or hedonic feeding) (Abizaid and Horvath, 2008; Berridge, 2009; Liu and Kanoski, 2018). In general, homeostatic processes work to regulate feeding in response to immediate energy requirements (e.g. low concentrations of glucose); whereas, non-homeostatic processes promote feeding based on the reinforcing value of foods (motivated reward-based feeding) (Abizaid and Horvath, 2008). It is important to note that non-homeostatic processes may be encouraged by energy-deficient states but can and often are engaged in the absence of them (Abizaid and Horvath, 2008; Berridge, 2009; Liu and Kanoski, 2018). Homeostatic feeding is primarily regulated by the hypothalamus (HYP) and brainstem, while the mesocorticolimbic dopamine system orchestrates non-homeostatic reward-based feeding (Saper et al., 2002; Berthoud, 2006; Liu and Kanoski, 2018).

The stomach-derived peptide ghrelin and endogenous cannabinoids (i.e. endocannabinoids) potently stimulate both homeostatic and non-homeostatic feeding (Kojima et al., 1999; Williams and Kirkham, 1999; Ariyasu et al., 2001; Cummings et al., 2001; Jamshidi and Taylor, 2001; Perio et al., 2001; Kirkham et al., 2002; Cowley et al., 2003; Naleid et al., 2005; Solinas and Goldberg, 2005; Abizaid et al., 2006b; Kirkham, 2009; Oleson et al., 2012). Interestingly, ghrelin and endocannabinoid systems appear to jointly drive homeostatic feeding within the HYP, as disruption of either system eliminates the orexigenic capacity of either ghrelin or endocannabinoids within this region (Kola et al., 2008; Lim et al., 2013; Edwards and Abizaid, 2016). In addition, there is some indirect evidence, that these systems may also

interact in other brain regions, such as the mesocorticolimbic dopaminergic system, to increase the motivation to eat (Edwards and Abizaid, 2016). The goal of the present thesis was to elucidate if and how these systems interact within the ventral tegmental area (VTA), the hub of the mesocorticolimbic dopamine system, to promote non-homeostatic feeding behaviours. Accordingly, Chapter 1 describes how ghrelin and endocannabinoids act within the periphery and the brain to influence feeding behaviours and metabolism and highlights accumulating evidence that ghrelin and endocannabinoid systems work together to do so, setting the stage for our hypothesis ***that ghrelin and endocannabinoid systems interact within the VTA and that this interaction is important for encouraging motivated feeding behaviours***. The ensuing chapters detail the molecular, behavioural, and electrophysiological experiments conducted to test our overarching hypothesis. Chapter 2 describes the molecular experiments performed to test whether ghrelin and endocannabinoid systems interact within the VTA. Chapter 3 outlines several behavioural studies designed to determine whether endocannabinoid signaling is likewise required for ghrelin to induce feeding within the VTA. Lastly, chapter 4 details electrophysiological experiments intended to elucidate the extent to and the mechanism by which ghrelin interacts with the endocannabinoid system to increase the activity of dopamine cells in the VTA, a cellular process associated with increased motivation to eat (Salamone et al., 1990, 1993; Richardson and Gratton, 1996; Saper et al., 2002; Abizaid et al., 2006b; Baler and Volkow, 2006). Together the proposed experiments aim to test the hypothesis that ghrelin and endocannabinoid systems collaboratively interact within the VTA to stimulate VTA dopamine cells that drive motivated feeding behaviours.

Chapter 1

Driving the need to feed: Insight into the collaborative interaction between ghrelin and endocannabinoid systems in modulating feeding behaviours

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1.1 Introduction

1.1.1 The “feeding peptide” ghrelin

Ghrelin is an appetite-stimulating, 28-amino-acid, peptide predominantly produced and secreted by endocrine gastric X/A-like cells of the oxyntic mucosa lining of the stomach (Kojima et al., 1999; Date et al., 2000; Ariyasu et al., 2001; Dornonville De La Cour et al., 2001).

Consistent with its designation as a feeding peptide, circulating ghrelin levels fluctuate with feeding status and are closely associated with scheduled meals; rising just before meal onset and declining after consumption (Ariyasu et al., 2001; Cummings et al., 2001; Dornonville De La Cour et al., 2001; Cummings, 2006; Drazen et al., 2006; Verbaeys et al., 2011). Ghrelin stimulates food intake and promotes adiposity by activating its endogenous receptor, the type 1a growth hormone secretagogue receptor (GHSR), which is highly expressed in feeding related brain regions (e.g. hypothalamus (HYP) and ventral tegmental area (VTA)) and peripheral

organs associated with energy balance (e.g. liver and adipose tissue) (Howard et al., 1996; Guan et al., 1997; Papotti et al., 2000; Tschop et al., 2000; Nakazato et al., 2001; Shuto et al., 2001; Wren et al., 2001b, 2001a; Gnanapavan et al., 2002; Sun et al., 2004; Zigman et al., 2006; Ferrini et al., 2009; Shrestha et al., 2009; Mani et al., 2014). Consistent with this, exogenous ghrelin induces feeding and promotes adiposity when administered peripherally (i.e. intraperitoneal or intravenous) or centrally (i.e. intracerebroventricular (ICV), intra-HYP, and intra-VTA) in both satiated and non-satiated rodents (Nakazato et al., 2001; Wren et al., 2001a, 2001b; Abizaid et al., 2006b; Cummings, 2006; Kageyama et al., 2010). The feeding and adipogenic effects induced by exogenously administered ghrelin are mediated by GHSRs as they are blocked by GHSR antagonists (Nakazato et al., 2001; Asakawa et al., 2003; Sun et al., 2004; Zigman et al., 2005; Abizaid et al., 2006b; Perello et al., 2010; Vodnik et al., 2016). Consistent with this, ghrelin administration does not induce feeding or weight gain in GHSR deficient mice (Sun et al., 2004; Perello et al., 2010). Importantly, tonic GHSR signaling is important for promoting food intake as antagonism of GHSRs in the absence of exogenous GHSR agonism reduces feeding compared to controls (Asakawa et al., 2003). Together, these data highlight that ghrelin robustly induces feeding and adiposity via GHSR-dependent processes.

Ghrelin uniquely requires a posttranslational modification, most commonly an octanoylation, on serine-3 of its peptide to stimulate GHSRs (Bednarek et al., 2000; Gutierrez et al., 2008; Yang et al., 2008). This reaction, which is mediated solely by ghrelin O-acyltransferase (GOAT), induces a conformational alteration in the peptide that permits efficient binding to the GHSR active site (Bednarek et al., 2000; Gutierrez et al., 2008; Yang et al., 2008). Like ghrelin, GOAT expression oscillates in response to feeding status and diet, with the highest levels found in energy depleted states (Drazen et al., 2006; Kirchner et al., 2009). GOAT messenger ribonucleic acids (mRNAs) are ubiquitously expressed within many peripheral organs but are typically highest in organs that also strongly express ghrelin mRNAs (e.g. stomach and intestines) (Guan et al., 1997; Gutierrez et al., 2008; Lim et al., 2011; Wellman and Abizaid, 2015). This suggests that ghrelin is likely converted into its active form (i.e. acyl-ghrelin) before it is secreted into the circulation. Conversely, there is also evidence that GOAT is expressed

within feeding related brain regions, such as the HYP, and may convert inactive ghrelin (i.e. des-acyl ghrelin) into active ghrelin centrally (Gahete et al., 2010; Wellman and Abizaid, 2015). Consistent with this, in high fat diet fed rats, central knockdown of GOAT expression reduces weight gain and caloric efficiency compared to control animals (Wellman and Abizaid, 2015).

Surprisingly, it is estimated that only 10% of circulating ghrelin is in an active acylated form capable of stimulating GHSRs; the rest circulates in a des-acyl ghrelin form that does not activate GHSRs at physiological relevant concentrations (Kojima et al., 1999; Hosoda et al., 2000; Patterson et al., 2005). Despite not being able to activate GHSRs, there have been reports that des-acyl ghrelin can influence feeding (Asakawa et al., 2005; Chen et al., 2005; Neary et al., 2006; Toshinai et al., 2006; Inhoff et al., 2008). While some report that des-acyl ghrelin decreases food intake when rodents are in their dark cycle or are food deprived (Asakawa et al., 2005; Chen et al., 2005), others find no effect of des-acyl ghrelin irrespective of feeding status or light cycle (Neary et al., 2006; Inhoff et al., 2008). Furthermore, some argue that des-acyl ghrelin can inhibit the orexigenic effect of ghrelin, while others suggest that des-acyl ghrelin administration increases feeding (Neary et al., 2006; Inhoff et al., 2008). It is evident that more work is needed to clarify the mechanism by which des-acyl ghrelin influences feeding; however, the consensus is that it does so through GHSR independent processes (van der Lely et al., 2004; Asakawa et al., 2005; Chen et al., 2005; Toshinai et al., 2006; Delhanty et al., 2012). Consistent with this, it has been proposed that des-acyl ghrelin may bind to a yet uncharacterised receptor to elicit its biological effects (e.g. cardiovascular, metabolic, anti-proliferative) (Hosoda et al., 2000; Broglio et al., 2004; van der Lely et al., 2004; Muccioli et al., 2007; Ferrini et al., 2009).

Given the robust ability of ghrelin to stimulate food intake and weight gain, it is not surprising that GHSRs are highly expressed within the HYP and VTA, the main brain regions that regulate homeostatic and non-homeostatic feeding, respectively (Guan et al., 1997; Abizaid et al., 2006b; Jiang et al., 2006; Zigman et al., 2006; Mani et al., 2014). In both brain regions, ghrelin-binding and electrophysiology studies suggest that activation of pre- and postsynaptic GHSRs engages neurocircuits that promote feeding (Howard et al., 1996; Guan et al., 1997;

Cowley et al., 2003; Kohno et al., 2003, 2008; Abizaid et al., 2006b; Zigman et al., 2006; Dickson et al., 2010; Jerlhag et al., 2011a; Yang et al., 2011). GHSRs are coupled to excitatory guanine nucleotide-binding proteins (G-proteins), which become activated following ghrelin binding (Yin et al., 2014). Accordingly, ghrelin-induced GHSR activation predominantly stimulates the $G\alpha_q$ subunit. $G\alpha_q$ stimulation promotes the production and activation of phospholipase C (PLC), which cleaves membrane associated phosphoinositol 4,5 diphosphate into inositol triphosphate (IP_3) and diacylglycerol (DAG) second messengers (Yin et al., 2014). IP_3 increases intracellular calcium stores from the endoplasmic reticulum, while DAG activates protein kinase C to inhibit potassium channels (Yin et al., 2014). Together these processes work to depolarize membranes and enhance the overall excitability of neurons (Yin et al., 2014). Consistent with this, GHSRs are commonly expressed on and activate neurons known to promote appetite and feeding behaviours (e.g. neuropeptide Y/agouti-related peptide/ gamma aminobutyric acid (NPY/AgRP/GABA) and dopaminergic neurons of the HYP and VTA, respectively) (Cowley et al., 2003; Abizaid et al., 2006b). Moreover, ghrelin-mediated activation of GHSRs within the HYP also induces changes in synaptic connections (i.e. alters excitatory and inhibitory afferent profiles) to indirectly promote activation of orexigenic circuits, whilst suppressing satiety promoting pathways (Nakazato et al., 2001; Cowley et al., 2003; Kohno et al., 2003; Pinto et al., 2004; Horvath, 2006; Abizaid and Horvath, 2012). Likewise, while ghrelin promotes feeding behaviours by activating GHSRs directly on VTA dopamine neurons, ghrelin also induces the rearrangement of synaptic inputs at these neurons in a manner that increases the probability of their activation (Naleid et al., 2005; Abizaid et al., 2006b). Once activated, these cells enhance their release of dopamine in efferent targets, such as the nucleus accumbens (NA), consequentially engaging motivational processes to seek and consume desired foods (Abizaid et al., 2006; Cone et al., 2015; Jerlhag et al., 2007; King et al., 2011; Van Der Plasse et al., 2015). The mechanisms by which ghrelin engages orexigenic circuits in the HYP and VTA will be discussed in more detail subsequently (see 1.3.2 and 1.4.4, respectively).

1.1.2 Endocannabinoids: lipid regulators of feeding

While the appetite inducing effects of *Cannabis sativa* have been recognized for centuries, the identification of Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the chemical responsible for a majority of the psychoactive and orexigenic effects, as well as the cloning of the first two endogenous receptors to which it binds (i.e. cannabinoid receptor 1 (CB-1R) and cannabinoid receptor 2 (CB-2R)), did not occur until much later (Gaoni and Mechoulam, 1964; Devane et al., 1988, 1992; Matsuda et al., 1990; Munro et al., 1993). Interestingly, it is now known that CB-1Rs are expressed predominantly in neurons and are arguably the most abundant G-protein-coupled receptor (GPCR) in the brain; whereas, CB-2Rs are found to a lesser extent in the brain, yet abundantly on immune cells and in peripheral organs (e.g. spleen, thymus, pancreas, etc.) (Herkenham et al., 1991; Howlett et al., 2002; Mackie, 2005; Svíženská et al., 2008). Endogenous cannabinoids, aptly named endocannabinoids, are the natural ligands of cannabinoid receptors. Arachidonic acid derivatives, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), were the first two endocannabinoids discovered and remain the two most extensively studied (Devane et al., 1992; Di Marzo and Fontana, 1995; Sugiura et al., 1995). 2-AG, which is 100–1000 fold more abundant than AEA, binds to both CB-1Rs and CB-2Rs with moderate affinity and is considered a full agonist at these receptors (Bisogno et al., 1999; Howlett et al., 2002). AEA on the other hand acts as a high affinity partial agonist at CB-1Rs but does not efficiently bind and activate CB-2Rs (Howlett et al., 2002). 2-AG and AEA are most commonly produced “on demand” from lipids of the postsynaptic membrane in response to intracellular increases in calcium induced by depolarization events (Wilson and Nicoll, 2001). 2-AG is synthesized from phospholipid precursors by the sequential activation of PLC and diacylglycerol lipase (DAGL) enzymes, while AEA is predominately produced by N-arachidonoyl phosphatidylethanolamine-phospholipase D (NAPE-PLD) (Basavarajappa, 2007; Liu et al., 2008; Murataeva et al., 2014). These endocannabinoid synthesizing enzymes are predominantly found perisynaptically in postsynaptic cell dendrites (Mátyás et al., 2008; Reguero et al., 2014). While many enzymes have been reported to degrade 2-AG and AEA, monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), are the two most predominant (Basavarajappa, 2007; Murataeva et al., 2014). MAGL, the enzyme that breaks down 2-AG, is found primarily in

CB-1R expressing presynaptic axon terminals, whereas FAAH, the enzyme that breaks down AEA and to a lesser extent 2-AG, is located postsynaptically in soma and dendrites (Gulyas et al., 2004). Consistent with the postsynaptic location of DAGL and NAPE-PLD biosynthetic enzymes, 2-AG and AEA predominantly travel in a retrograde manner from postsynaptic neurons to presynaptic axon terminals, where they bind inhibitory $G_{i/o}$ -coupled CB-1Rs to discourage neurotransmitter release (Ohno-Shosaku et al., 2001; Schlicker and Kathmann, 2001; Alger, 2002). However, recent evidence has shown that CB-2Rs are also expressed on the postsynaptic cell membrane of neurons and that activation of these receptors consequentially initiates $G_{i/o}$ -coupled signaling cascades to decrease neuronal excitability (Zhang et al., 2014; Ma et al., 2019). Moreover, it is becoming increasingly clear that endocannabinoids do not solely bind CB-1 and CB-2 receptors. They also activate other receptors such as the orphan G-protein-coupled receptor-55 (GPR55) and the transient receptor potential cation channel subfamily V member 1 (TRP-V1) (Ryberg et al., 2007; Kano et al., 2009; Grueter et al., 2010; Zygmunt et al., 2013). Interestingly, in some cases, it seems that AEA-induced activation of TRP-V1, can antagonize endocannabinoid signaling and encourage neural excitability (Maccarrone et al., 2008; Musella et al., 2009). Nevertheless, given that CB-1R expression is far more ubiquitous than TRP-V1 expression in the brain and that basal levels of 2-AG vastly exceed those of AEA, it is largely assumed that endogenous endocannabinoid release predominantly stimulates presynaptic CB-1Rs to dampen neurotransmitter release (Bisogno et al., 1999; Alger, 2002; Svíženská et al., 2008; Cavanaugh et al., 2011). Together, 2-AG and AEA, CB-1 and CB-2 receptors, and the enzymes involved in the synthesis and degradation of these endocannabinoids collectively make up the endocannabinoid system (D'Addario et al., 2014; Edwards and Abizaid, 2016).

Consistent with the idea that endocannabinoids are important regulators of feeding, their levels oscillate with feeding status (i.e. peak in the fasted state and fall shortly after meal consumption) (Kirkham et al., 2002). Like ghrelin, 2-AG and AEA also potently stimulate feeding behaviours by activating their receptors (i.e. CB-1Rs) within feeding related brain regions (e.g. HYP and VTA) (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Tsou et al., 1998; Marsicano and Lutz, 1999; Williams and Kirkham, 1999; Jamshidi and

Taylor, 2001; Perio et al., 2001; Solinas and Goldberg, 2005; Wittmann et al., 2007; Mátyás et al., 2008; Oleson et al., 2012). Accordingly, peripheral or central administration of 2-AG or AEA induces robust feeding behaviours and weight gain (Hao et al., 2000; Jamshidi and Taylor, 2001; Kirkham et al., 2002; Williams and Kirkham, 2002). Importantly, these endocannabinoid-induced effects are CB-1R mediated as they can be occluded by pretreating animals with CB-1R antagonists and are not observed in CB-1R knockout (KO) animals (Hao et al., 2000; Jamshidi and Taylor, 2001; Kirkham et al., 2002; Williams and Kirkham, 2002; Kola et al., 2008; Bellocchio et al., 2010). In addition, it seems that tonic endocannabinoid signaling is important for regulating feeding and adiposity as administration of CB-1R antagonists in the absence of exogenous CB-1R agonism reduces both feeding and adiposity in both satiated and non-satiated rodents (Arnone et al., 1997; Colombo et al., 1998; Werner and Koch, 2003; Senin et al., 2013). Together, these data highlight that endocannabinoids promote feeding through activation of central CB-1Rs.

Although, expression of CB-1R mRNA is relatively low within the HYP and VTA, CB-1R protein is present throughout these brain regions (Herkenham et al., 1991; Matsuda et al., 1993; Marsicano and Lutz, 1999; Wittmann et al., 2007; Mátyás et al., 2008). Consistent with the canonical retrograde signaling of endocannabinoids, CB-1R proteins are predominately found on axon terminals innervating these brain regions and not necessarily on the principal neurons traditionally thought to drive feeding (Wittmann et al., 2007; Mátyás et al., 2008) but see (Koch et al., 2015). The spatial separation of CB-1R mRNA, located in nuclei of neurons which project to the HYP and VTA, and protein, situated on axon terminals innervating these nuclei, may partially explain the apparent disparity between CB-1R mRNA and protein expression studies (Wittmann et al., 2007; Mátyás et al., 2008). Nonetheless, CB-1R agonism within the HYP or VTA promotes homeostatic and non-homeostatic feeding, respectively. Like GHSR activation, stimulation of CB-1Rs within the HYP alters excitatory and inhibitory afferent profiles to promote the activity of neurons that stimulate feeding and inhibit neurons that normally signal satiety (Kola et al., 2008; Verty et al., 2009; Morozov et al., 2017). Similarly, activation of CB-1Rs within the VTA stimulates dopaminergic neurons within this region,

augments dopamine release within the NA, and encourages associated non-homeostatic feeding behaviours (Chen et al., 1990; French, 1997; French et al., 1997; Gifford et al., 1997; Tanda et al., 1997; Gessa et al., 1998; Cheer et al., 2000; Solinas et al., 2006). The role of the endocannabinoid system in modulating feeding within these two regions will be discussed in more detail subsequently.

1.2 Peripheral interactions of ghrelin and endocannabinoid systems

Given the functional overlap between ghrelin and endocannabinoid systems in promoting food intake and adiposity and the co-expression of their respective receptors in peripheral organs associated with metabolism (e.g. stomach, liver, adipose tissue), it is not surprising that these systems influence each others ability to regulate energy balance (Herkenham et al., 1991; Guan et al., 1997; Hao et al., 2000; Tschop et al., 2000; Wren et al., 2001b; Jamshidi and Taylor, 2001; Wren et al., 2001a; Gnanapavan et al., 2002; Kirkham et al., 2002; Williams and Kirkham, 2002; Zigman et al., 2006; Mani et al., 2014).

1.2.1 Ghrelin and endocannabinoid systems promote adiposity by inhibiting AMPK signaling cascades in adipose tissue and the liver

Ghrelin and endocannabinoid systems similarly regulate adenosine monophosphate activated protein kinase (AMPK), an important intracellular regulator of energy metabolism and appetite, in adipose tissue and the liver (Kola et al., 2005). AMPK acts as a resource sensor and regulates energy balance at both the cellular and whole-body level (Hardie et al., 2006). AMPK is activated when energy levels are low (i.e. high adenosine monophosphate (AMP): adenosine triphosphate (ATP) ratio) and inhibited when energy stores are high (i.e. low AMP: ATP ratio) (Hardie and Carling, 1997). Activation of AMPK signaling shifts intracellular activity away from anabolic pathways that further deplete ATP in favour of ATP-producing pathways (Hardie and

Carling, 1997; Hardie et al., 1998, 2006). Accordingly, once activated, AMPK increases fatty acid oxidation and glycolysis and prevents the synthesis of fatty acids and glycogen (Hardie et al., 2006). In agreement with the ability of ghrelin and endocannabinoid treatments to promote lipid conservation and weight gain, activation of either ghrelin or endocannabinoid systems inhibits adipose tissue and liver AMPK activity (Kola et al., 2005). This inhibition results in a consequential decrease in the utilization of fat and glycogen and ultimately promotes adiposity (Tschop et al., 2000; Cota et al., 2003; Kola et al., 2005; Osei-Hyiaman et al., 2005; Daval et al., 2006; Kola, 2008). Interestingly, ghrelin and endocannabinoid systems are mutually dependent on each other with respect to their capacity to inhibit liver and adipose AMPK signaling cascades (Kola et al., 2013; Lim et al., 2013). Peripheral administration of ghrelin at doses known to potently inhibit adipose tissue and liver AMPK activity are ineffective at doing so in rimonabant-treated or CB-1R KO mice (Kola et al., 2013). Likewise, the traditional ability of cannabinoids to inhibit AMPK activity within these peripheral tissues is absent in GHSR KO mice (Lim et al., 2013). Consequently, despite similar energy intake, CB-1R and GHSR/ghrelin double KO mice gain less weight and fat compared to corresponding controls as they are unable to attenuated adipose tissue and liver AMPK activity and associated catabolic processes (Ravinet Trillou et al., 2004; Pfluger et al., 2008). These studies not only highlight that ghrelin and endocannabinoid systems interact within adipose tissue and the liver, they also suggest that the adipogenic effects elicited by either ghrelin or endocannabinoids are dependent on each other and involve the inhibition of AMPK signaling cascades.

1.2.2 Endocannabinoid system positively regulates ghrelin secretion

The stomach, which highly expresses both GHSRs and CB-1Rs, is another peripheral organ in which these two systems interact (Kojima et al., 1999; Date et al., 2000; Cani et al., 2004; Zbucki et al., 2008; Senin et al., 2013). The endocannabinoid system positively regulates ghrelin secretion from the stomach as exemplified by concomitant reductions in ghrelin-immunoreactivity in the gastric mucosa and heightened circulating ghrelin levels following peripheral CB-1R agonist administration (Zbucki et al., 2008). Furthermore, CB-1R signaling is involved in hunger-induced ghrelin release and feeding as food deprived rats treated with CB-

1R antagonists demonstrate quick (i.e. 15 minutes) and significant reductions in plasma ghrelin and blunted re-feeding compared to vehicle treated animals (Cani et al., 2004; Senin et al., 2013). Although no one has looked at the impact that GHSR antagonism has on circulating endocannabinoid levels, endocannabinoid concentrations within feeding related brain regions (e.g. HYP) increase when ghrelin levels are high (i.e. in the fasted state or with exogenous ghrelin administration) (Kola et al., 2008; Kirkham et al., 2002).

1.2.3 Interaction of ghrelin and endocannabinoid systems within the nodose ganglia influences feeding

Blockage of peripheral CB-1Rs, using peripherally restricted CB-1R antagonists or extremely low doses of rimonabant (thought to only activate peripheral CB-1Rs), attenuates the orexigenic capacity of ICV ghrelin (Alen et al., 2013). This suggests that peripheral interactions between ghrelin and endocannabinoid systems likewise influence the orexigenic action of ghrelin in the brain (Alen et al., 2013). The vagus nerve (i.e. tenth cranial nerve) is integral for brain-gut communication as it mediates the transmission of neural signals from the brain to the gastrointestinal system and likewise conducts hormonal information from the gastroenteric system to the brain (Sawchenko, 1983; van der Kooy et al., 1984; Morton et al., 2006). Although the vagus nerve is comprised of both afferent and efferent nerve fibers, approximately 90% of the neurons are afferent connections to the nucleus of the solitary tract (Agostoni et al., 1957). The nodose ganglia, which houses these vagal afferent cell bodies, has been identified as a peripheral location where ghrelin and endocannabinoid systems likely interact (Burdyga et al., 2006; Alen et al., 2013; Senin et al., 2013). Accordingly, GHSRs and CB-1Rs are highly expressed within the nodose ganglia and functional vagal afferents have been reported to be important for peripherally administered ghrelin and CB-1R agonist to increase and for CB-1R antagonists to decrease feeding (Date et al., 2002; Gomez et al., 2002; Burdyga et al., 2006; Senin et al., 2013). In support of an interaction between these systems within the nodose ganglia, these systems modulate the signaling capacity of one another within this region. Peripheral ghrelin increases the expression of CB-1Rs within the nodose ganglia, while destroying this region prevents the reduction in gastric ghrelin secretion that is traditionally observed with peripheral

rimonabant administration (Burdyga et al., 2006; Senin et al., 2013). Furthermore, although peripheral rimonabant administration attenuates the rise in food intake that accompanies central injections of ghrelin, this effect is not observed in vagotomised rats (Senin et al., 2013). Although these studies suggest that ghrelin and endocannabinoid systems require an intact vagus connection, studies that specifically interrogate where and how ghrelin and endocannabinoid systems interact at the level of the vagus nerve are required.

1.3 Ghrelin and endocannabinoid systems: homeostatic modulators of feeding

1.3.1 The hypothalamus (HYP): master homeostatic regulator of feeding

Although many brain regions contribute to the regulation of feeding and energy balance, the HYP is largely considered to be the “master regulator of homeostatic feeding” (Timper and Bruning, 2017). The HYP has been attributed this designation for several reasons. First, lesion of any one of the five nuclei that make up the HYP including the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), or arcuate nucleus (ARC), perturbs normal feeding behaviours and makes animals either hyperphagic (PVN, VMN, and ARC lesions) or hypophagic (DMN and LHA lesions) (Hetherington and Ranson, 1940; Brobeck et al., 1943; Hetherington, 1944; Anand and Brobeck, 1951). Second, the HYP, which bilaterally borders the third ventricle at the base of the diencephalon, shares its ventromedial border (i.e. ARC) with the median eminence (a circumventricular organ) and hence has privileged access to many circulating energy related signals (Cheunsuang and Morris, 2005; Cheunsuang et al., 2006; Münzberg, 2008; Norsted et al., 2008; Schaeffer et al., 2013). Accordingly, the ARC of the HYP has receptors for and is sensitive to most hormones and nutrients that modulate feeding and energy balance, including ghrelin and endocannabinoids (Herkenham et al., 1991; Matsuda et al., 1993; Kirkham et al.,

2002; Cowley et al., 2003; Leibowitz and Wortley, 2004; Cheunsuang and Morris, 2005; Abizaid et al., 2006a; Cheunsuang et al., 2006; Norsted et al., 2008; Münzberg, 2008; Suzuki et al., 2012; Duarte et al., 2012; Schaeffer et al., 2013). The independent roles of ghrelin and endocannabinoid systems in promoting orexigenic circuits within the HYP will be discussed briefly before evidence of their interaction and its importance in mediating these processes is described.

1.3.2 The orexigenic action of ghrelin within the HYP

Despite the fact that all five major hypothalamic nuclei express GHSRs and demonstrate the capacity to initiate food intake in response to local ghrelin microinfusion, the focus will remain on the actions of ghrelin within the ARC as this nucleus is directly influenced by endogenous ghrelin and the neurocircuits that transduce the orexigenic action of ghrelin within the ARC have been the best characterized (Luckman et al., 1999; Hewson and Dickson, 2000; Cowley et al., 2003; Olszewski et al., 2003; Zigman et al., 2006; Scott et al., 2007; Currie et al., 2012; Schaeffer et al., 2013; Mani et al., 2014). Consistent with the importance of the ARC in mediating ghrelin-induced feeding, ARC lesions abolish rises in food consumption that traditionally occur following central or peripheral ghrelin administration (Tamura et al., 2002; Bugarith et al., 2005).

Appropriately, GHSRs are expressed in the two major but opposing first-order energy sensing neurons that regulate homeostatic feeding within the ARC: NPY/AgRP/GABA and proopiomelanocortin/cocaine and amphetamine regulated transcript (POMC/CART) neurons (Willesen et al., 1999). NPY/AgRP/GABA neurons enhance appetite when activated, whereas POMC/CART neurons promote satiety when stimulated (Boston et al., 1997; Hahn et al., 1998; Cowley et al., 2003; Ellacott and Cone, 2004; Aponte et al., 2011; Dietrich and Horvath, 2013). Central ghrelin administration upregulates the expression of orexigenic peptide transcripts, NPY and AgRP, but does not influence the expression of the anorectic peptide POMC within the ARC (Kamegai et al., 2001). Accordingly, although ghrelin-binding studies show that ghrelin targets both cell types, the direct action of ghrelin at GHSR-positive POMC/CART neurons has not been

characterized to the same extent (Cowley et al., 2003; Schaeffer et al., 2013). Ghrelin enhances the activity of NPY/AgRP/GABA neurons by both binding to GHSRs on their surface and by increasing the ratio of excitatory to inhibitory afferents contacting them (Cowley et al., 2003; Riediger et al., 2003; Horvath, 2006; Dietrich and Horvath, 2013). As one would expect, the proportion of NPY/AgRP/GABA neurons activated by ghrelin is higher during times of energy insufficiency when endogenous ghrelin levels are high and food procuring behaviours are needed (Schaeffer et al., 2013). Conversely, ghrelin inhibits POMC/CART neurons by decreasing the proportion of excitatory to inhibitory afferents innervating them and by stimulating GABA release from NPY/AgRP/GABA onto them (Cowley et al., 2003; Riediger et al., 2003; Horvath, 2006; Dietrich and Horvath, 2013). Once activated NPY/AgRP/GABA neurons stimulate feeding by increasing the production and release of NPY, AgRP, and GABA, which together act to inhibit satiety promoting pathways (Stanley and Leibowitz, 1984; Mountjoy et al., 1994; Ollmann et al., 1997; Cowley et al., 2003; Tong et al., 2008). As mentioned, GABA released from NPY/AgRP/GABA neurons directly inhibits neighbouring satiety promoting POMC/CART neurons (Cowley et al., 2003). Concurrently, AgRP released from NPY/AgRP/GABA neurons antagonizes the binding of POMC/CART neuron released α -melanocyte-stimulating hormone (α -MSH) and β -melanocyte-stimulating hormone (β -MSH) at melanocortin receptors (i.e. MC-4Rs) located in the PVN, LHA, and DMN, to dampen anorectic outputs from these regions (Adan et al., 1994; Mountjoy et al., 1994; Sahm et al., 1994; Ollmann et al., 1997; Elias et al., 1999; Biebermann et al., 2006; Aponte et al., 2011). Lastly, the enhanced production and release of NPY from NPY/AgRP/GABA projections innervating many other hypothalamic nuclei (i.e., PVN, VMN, and LHA) also encourages appetite (Stanley and Leibowitz, 1984; Mountjoy et al., 1994; Sahm et al., 1994; Baskin et al., 1999; Gao and Horvath, 2007; Abizaid and Horvath, 2008). On the other hand, ghrelin levels drop shortly following food consumption, whereas nutrient and hormonal satiety signals rise (Dietrich and Horvath, 2013). Consequently, NPY/AgRP/GABA neuron activity slows down resulting in reduced inhibitory (i.e., reduced GABA tone) control over POMC/CART neurons and reduced NPY production and secretion (Cowley et al., 2003). In addition, POMC/CART neuron secreted anorectic peptides, α -MSH and β -MSH, are less antagonized by AgRP at melanocortin receptors in the HYP (Abizaid and Horvath, 2008). Ultimately, the

heightened production of α -MSH, β -MSH, and CART from POMC neurons and reduced NPY/AgRP/GABAergic activity leads to the dampening of orexigenic pathways and the manifestation of satiety (Abizaid and Horvath, 2008).

Recent evidence has begun to uncover the intracellular signaling cascades engaged after ghrelin activates GHSRs and the importance that these processes have in transducing the orexigenic signal of ghrelin within the HYP. One of the most important consequences of ghrelin-mediated GHSR activation is the stimulation of AMPK. Not surprisingly, similar to GOAT expression and ghrelin secretion, hypothalamic AMPK activity also fluctuates with feeding status (i.e. highest in fasted state and lowest following re-feeding) (Minokoshi et al., 2004). Similarly, like ghrelin, AMPK activators and expression of constitutively active AMPK within the HYP increases the expression of orexigenic peptides (i.e. NPY and AgRP), food intake, and body weight; whereas, knocking down AMPK activity induces the opposite effects (Andersson et al., 2004; Minokoshi et al., 2004). Signals that convey energy sufficiency such as high blood glucose levels and anorexigenic peptides (e.g. leptin and insulin) negatively regulate hypothalamic AMPK activity, while signals that convey energy insufficiency, such as low blood glucose and orexigenic compounds (e.g. ghrelin and endocannabinoids), stimulate it (Xue and Kahn, 2006; Kola et al., 2008). In support of this, exogenous administration of ghrelin, at a dose that increases food intake, enhances the activity of AMPK within the HYP; whereas, pharmacological inhibition of AMPK abolishes the orexigenic capacity of ghrelin (Andersson et al., 2004; Kola et al., 2005; Andrews et al., 2008; López et al., 2008). Consistent with this, HYP AMPK activity and feeding are unaltered in GHSR deficient mice following ghrelin administration suggesting that activation of AMPK activity is downstream of GHSR stimulation and necessary for ghrelin-induced feeding (Sun et al., 2004; Andrews et al., 2008; López et al., 2008; Perello et al., 2010). It is thought that ghrelin predominantly stimulates AMPK activity by stimulating $G\alpha_q$ associated GHSRs; however, GHSR- $G\alpha_s$ dependent mechanisms that engage cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling cascades have also been reported (Collins et al., 2000; Kohno et al., 2003; Granata et al., 2007; Hutchinson et al., 2008; Andrews, 2011). $G\alpha_q$ subunit activation initiates PLC/IP₃ signaling cascades that consequently heighten

intracellular calcium concentrations to levels that trigger calcium/calmodulin-dependent protein kinase II (CaMKII) activity (Kola et al., 2008; López et al., 2008). Activation of this kinase leads to enhanced phosphorylation and activation of AMPK (Kola et al., 2008; López et al., 2008). Collectively, these data highlight that ghrelin-mediated GHSR activation enhances the activity of AMPK within the HYP to promote homeostatic feeding.

The signaling cascades initiated downstream of AMPK activation are complex and speculative as they have been worked out using different sub-nuclei of the HYP or the HYP in its entirety. Although this limits one's ability to state that ghrelin is inducing AMPK signaling cascades solely in neurons that promote feeding and does not allow for the precise identification of these neurons, the fact that many independent research groups have largely uncovered the same signaling pathway demonstrates the importance and robust nature of this signaling cascade in conveying the orexigenic message of ghrelin following GHSR activation within the HYP (Obici et al., 2002, 2003; Andersson et al., 2004; Kola et al., 2005, 2008; Lam et al., 2005; Andrews et al., 2008; López et al., 2008; Andrews, 2011; Edwards and Abizaid, 2016). Importantly, the ability of ghrelin to induce these AMPK signaling cascades specifically in neurons known to drive feeding, such as NPY/AgRP neurons, has been confirmed (Andrews et al., 2008; Kohno et al., 2008). GHSR-activated AMPK signaling cascades promote nutrient-sensitive fatty acid biosynthetic pathways within the HYP (Lam et al., 2005; Andrews, 2011). These cascades are necessary for the initiation and maintenance of cellular processes that drive orexigenic circuits (Lam et al., 2005; Andrews, 2011). Appropriately, uncoupling protein 2 (UCP-2), an inner mitochondrial membrane protein involved in bioenergetic cellular processes, was identified as an essential downstream enzyme required for the orexigenic effects of ghrelin (Andrews et al., 2008). In support of this, UCP-2 KO animals do not demonstrate an increase in hypothalamic NPY and AgRP mRNA expression and consume significantly less food than corresponding wild types (WTs) following peripheral ghrelin administration (Andrews et al., 2008). UCP-2 seems to be important for buffering against the increased reactive oxygen species that accompany ghrelin-induced fatty acid oxidation in NPY/AgRP neurons (Andrews et al., 2008). Andrews and colleagues (2008) argue that ghrelin induced increases in UCP-2 allow

these neurons to increase mitochondrial respiration and biogenesis to generate the ATP required for sustaining the activity of NPY/AgRP neurons within the ARC during periods of energy insufficiency.

1.3.3 The orexigenic action of endocannabinoids within the HYP

The endocannabinoid system plays an important role in the regulation of homeostatic feeding within the HYP. This is evidenced by the altered expression of hypothalamic feeding peptides and aberrant feeding behaviours that are observed following genetic or pharmacological manipulation of this system (Verty et al., 2009; Koch et al., 2015; Morozov et al., 2017). Appropriately, CB-1R mRNA and protein are detectable throughout the HYP and administration of CB-1R agonists and antagonists into various nuclei of the HYP increases and decreases food consumption, respectively (Herkenham et al., 1991; Jamshidi and Taylor, 2001; Verty et al., 2005; Wittmann et al., 2007; Koch et al., 2015). Moreover, endocannabinoid levels within the HYP oscillate with respect to the nutritional status of rodents (i.e. high in fasted states and low in satiated states) and are influenced by energy related peripheral hormonal signals, such as leptin and ghrelin (Di Marzo et al., 2001; Kirkham et al., 2002; Kola et al., 2008). Accordingly, hypothalamic endocannabinoid levels are negatively regulated by the anorectic peptide leptin and positively regulated by the appetite stimulating peptide ghrelin (Di Marzo et al., 2001; Kola et al., 2008). Along the same lines, chronic perturbations in energy homeostasis, as seen in mice with diet induced obesity and those with dysfunctional leptin receptors, also induces elevated hypothalamic endocannabinoid levels (Di Marzo et al., 2001; Ravinet Trillou et al., 2004). As a matter of fact, chronic activation of the endocannabinoid system within the HYP has been suggested to contribute to the increased appetite and food intake observed in these mice (Di Marzo et al., 2001; Ravinet Trillou et al., 2004). Together these data strongly support that endocannabinoids target CB-1Rs within the HYP to promote hunger driven food intake.

Despite significant evidence that CB-1R agonists promote feeding and that CB-1R antagonists reduce food intake within the HYP, the specific neurocircuits engaged and mechanisms responsible for regulating endocannabinoid-induced feeding behaviours have

been poorly characterized (Jamshidi and Taylor, 2001; Verty et al., 2005; Koch et al., 2015). Until recently, it was largely presumed that CB-1Rs were exclusively found on axons terminals of presynaptic neurons innervating hypothalamic nuclei (Wittmann et al., 2007). Therefore, it was thought that endocannabinoids must promote feeding by inhibiting neurotransmitter release from excitatory and inhibitory afferents to ultimately stimulate the activity of orexigenic neurons and/or reduce the activity of satiety promoting neurons within the HYP. Appropriately, within the ARC, axon terminals expressing CB-1Rs have been found apposed to the two opposing and most renowned first-order sensory cell populations: the orexigenic NPY/AgRP/GABA and anorexigenic POMC/CART neurons (Koch et al., 2015; Morozov et al., 2017). Interestingly, CB-1R-positive GABAergic but not CB-1R-positive glutamatergic terminals are evidenced to strongly innervate NPY/AgRP/GABA neurons (Morozov et al., 2017). Under conditions of fasting, when hypothalamic endocannabinoid levels are high, one would predict that CB-1R activation would disinhibit NPY/AgRP/GABA neurons and promote orexigenic pathways by mitigating GABA release onto these neurons. While this is purported to be one of the ways that endocannabinoids ultimately promote feeding within the HYP (Morozov et al., 2017), POMC/CART neurons are also more densely innervated by CB-1R-positive GABAergic terminals (Hentges et al., 2005; Koch et al., 2015). Consistent with this, CB-1R antagonists increase GABA_A-mediated evoked inhibitory postsynaptic currents (eIPSCs) in POMC/CART neurons while CB-1R agonists have been shown to promote the activity of POMC/CART neurons (Hentges et al., 2005; Koch et al., 2015). Clearly, CB-1Rs are well positioned to modulate the excitability of both NPY/AgRP/GABA and POMC/CART neuron populations. More research is needed to elucidate whether the endocannabinoid system can separately and selectively activate these neuron populations in different nutritional states to maintain energy homeostasis.

In addition to the presynaptic distribution of CB-1Rs within the ARC, it has also recently come to light that POMC/CART neurons express CB-1Rs on their postsynaptic membrane as well as in their mitochondria (Koch et al., 2015; Morello et al., 2016). Although counterintuitive, it has recently been demonstrated that peripheral CB-1R agonism increases the activity of

POMC/CART cells within the ARC and that this increase is essential for the traditional orexigenic effect of CB-1R agonism (Koch et al., 2015). Accordingly, Designer-Receptors-Exclusively-Activated-by-Designer-Drugs (DREADD)-mediated excitation of POMC/CART neurons enhanced, whereas DREADD-mediated inhibition of POMC/CART neurons abolished the feeding effects of peripherally administered CB-1R agonists (Koch et al., 2015). Interestingly, although the *Pomc* gene is most commonly associated with its ability to produce the anorexigenic peptide α -MSH, it also encodes the orexigenic peptide β -endorphin (Dores and Baron, 2011). The authors of this study argue that CB-1R agonists increase POMC/CART neuron activity and activate mitochondrial CB-1Rs and UCP-2 to selectively increase the release of β -endorphin from terminals in the PVN to promote feeding (Grandison and Guidotti, 1977; Cooper and Sanger, 1984; Koch et al., 2015). In contrast, others suggest that endocannabinoids produced by POMC cells act in an autocrine manner to reduce the expression of α -MSH and thus promote feeding behaviours by dampening satiety (Morello et al., 2016). Although the mechanisms by which the endocannabinoid system promotes feeding behaviours within the ARC are starting to emerge, more research is required to determine the canonical way in which this system does so.

The activation of AMPK signaling cascades within the HYP appears to be essential for the manifestation of feeding behaviours induced by orexigenic compounds (Andersson et al., 2004; Minokoshi et al., 2004). Endocannabinoids potently stimulate feeding within the HYP as evidenced by the robust feeding elicited when CB-1R agonists are injected into various hypothalamic regions (Jamshidi and Taylor, 2001; Tucci et al., 2004; Kola et al., 2005, 2008; Lim et al., 2013). As with GHSR stimulation, administration of CB-1R agonists at doses that potently stimulate feeding likewise heighten AMPK activity within the HYP (Kola et al., 2005; Kola, 2008; Kola and Korbonits, 2009; Lim et al., 2013). In contrast, peripheral CB-1R antagonism reduces the activation of hypothalamic AMPK and reduces feeding relative to controls (Hao et al., 2000; Kola et al., 2008). Interestingly, CB-1R agonists do not promote feeding or heighten hypothalamic AMPK activity in CB-1R deficient mice highlighting that endocannabinoids stimulate hypothalamic AMPK activity via a CB-1R dependent mechanism (Kola et al., 2008;

Bellocchio et al., 2010). The mechanism by which the endocannabinoid system engages hypothalamic AMPK activity remains largely unclear (Kola et al., 2005, 2008; Lim et al., 2013).

CB-1Rs are GPCRs that influence a plethora of different signaling cascades when activated (e.g. mitogen activated protein kinase (MAPK), cAMP-PKA, phosphatidylinositol 3-kinases) (Bouaboula et al., 1995; Mackie et al., 1995; Pertwee, 1997; Twitchell et al., 1997; Sanchez et al., 1998; Kano et al., 2009). The signaling cascades initiated following CB-1R activation depend largely on the identity of the G-protein coupled to the CB-1R (Bosier et al., 2010). Most predominately, CB-1Rs associate with the $G_{i/o}$ family of G-proteins (Howlett et al., 2010). When coupled with $G_{i/o}$ proteins, CB-1R stimulation leads to the activation of inwardly rectifying K^+ currents and MAPK cascades and the inhibition of voltage-gated Ca^{2+} channels and adenylyl cyclase (AC) (Howlett, 1985; Houston and Howlett, 1998; Howlett et al., 2002; Kano et al., 2009; Bosier et al., 2010). These CB-1R mediated effects are not consistent with those known to activate AMPK (Omar et al., 2009; Lim et al., 2010; Hardie, 2013). In contrast, activation of CB-1Rs in some instances has been reported to increase intracellular Ca^{2+} levels via a $G_{i/o}$ -initiated PLC/IP₃ signaling cascade (Sugiura et al., 1996, 1997; Filipeanu et al., 1997; Netzeband et al., 1999; De Petrocellis et al., 2007). Furthermore, albeit under specific conditions (e.g. high CB-1R agonist concentrations, presence of forskolin or pertussis toxin, association with other GPCRs), activation of CB-1Rs can also stimulate AC and downstream cAMP-PKA signaling cascades through G_s GPCR mechanisms (Glass and Felder, 1997; Maneuf and Brotchie, 1997; Abadji et al., 1999; Calandra et al., 1999; Rubovitch et al., 2002; Bash et al., 2003; Jarrahian et al., 2004; Kearn et al., 2005; Howlett et al., 2010). Endocannabinoids within the HYP may promote AMPK activation by engaging CB-1Rs associated with $G_{i/o}$ and G_s as both heightened intracellular Ca^{2+} levels and cAMP-PKA signaling cascades are known to promote AMPK activation; however, this has yet to be explored (Hawley et al., 2003, 2005; Woods et al., 2005; Lim et al., 2010; Hardie, 2013).

Although these processes may contribute, since canonical CB-1R-mediated signaling cascades are not suggested to directly activate AMPK activity, it is likely that the

endocannabinoid system indirectly effects AMPK activation within the HYP. One possibility, granted the ubiquitous expression of CB-1Rs on axon terminals innervating many hypothalamic nuclei and the ability of endocannabinoids to inhibit neurotransmitter release, is that endocannabinoids modulate synaptic afferents to these nuclei to indirectly influence AMPK activity (Wittmann et al., 2007; Kola et al., 2008). Given the inherent functional divisions within the HYP, it would not be surprising to see cell type specific alterations in AMPK activity as suggested by others (Minokoshi et al., 2004; Kola et al., 2008). Unfortunately, since studies investigating the impact of endocannabinoids on hypothalamic AMPK activity have either used the whole HYP or specific sub-nuclei, it remains unclear whether CB-1R-mediated alterations in AMPK activity is consistent between or within nuclei of the HYP (Kola et al., 2005, 2008; Lim et al., 2013). Future studies that examine how endocannabinoids influence AMPK activity in specific nuclei and cell populations will clarify the mechanism by which the endocannabinoid system influences hypothalamic AMPK activity to modulate feeding behaviours.

1.3.4 Hypothalamic feeding jointly regulated by ghrelin and endocannabinoid systems

Given that ghrelin and endocannabinoid systems have overlapping functional roles within the HYP (i.e. stimulate hypothalamic AMPK activity and promote feeding behaviours), it is not surprising that evidence of their interaction within this region has emerged (Herkenham et al., 1991; Guan et al., 1997; Ariyasu et al., 2001; Nakazato et al., 2001; Wren et al., 2001b; Jamshidi and Taylor, 2001; Kirkham et al., 2002; Cowley et al., 2003; Cummings and Foster, 2003; Tucci et al., 2004; Mackie, 2005; Verty et al., 2005; Zigman et al., 2006; Drazen et al., 2006; Wittmann et al., 2007; Kola et al., 2008; Kola and Korbonits, 2009; Lim et al., 2010, 2013).

Tucci and colleagues (2004) provided the first substantial evidence of this interaction as they demonstrated that a subanorectic dose of a CB-1R antagonist (rimonabant) abolished the orexigenic action of ghrelin when it was administered into the PVN. Follow-up studies determined that ghrelin is unable to stimulate hypothalamic AMPK activity, an event necessary

for the manifestation of the appetite-inducing effects of ghrelin, when CB-1Rs are either genetically or pharmacologically disrupted (Kola et al., 2008). This holds true when ghrelin is administered either peripherally (i.e. intraperitoneal injection) or centrally (i.e. ICV injection) (Kola et al., 2008). Interestingly, the traditional rise in hypothalamic 2-AG content that is observed following peripheral ghrelin injection is also abolished in CB-1R KO mice and animals treated with rimonabant (Kola et al., 2008). Collectively, these studies suggest that CB-1R activation is required for the ghrelin-mediated elevation of hypothalamic endocannabinoid levels, stimulation of AMPK signaling cascades, and induction of consequential feeding behaviours.

Similarly, the orexigenic capacity of CB-1R agonists within the HYP appear to rely on GHSR signaling. Accordingly, while intraperitoneal HU-210 (CB-1R agonist) injections tend to increase food intake in WT mice, they do not in GHSR-KO mice (Lim et al., 2013). Moreover, although CB-1R agonism increases hypothalamic AMPK activation in WTs, its ability to do so is abolished in mice with deficient GHSR signaling (Lim et al., 2013). Together this suggests that an intact ghrelin signaling pathway is required for endocannabinoids to initiate orexigenic neurocircuits within the HYP that are important for driving feeding behaviours.

Aside from their mutual promotion of AMPK activity within the HYP, the specific neurocircuits and mechanisms by which ghrelin and endocannabinoid systems jointly modulate feeding are relatively unknown. Electrophysiology experiments have identified the PVN as one of the hypothalamic regions in which ghrelin and endocannabinoid systems interact to modulate feeding (Kola et al., 2008). Within the PVN, ghrelin is known to inhibit excitatory tone onto satiety promoting corticotrophin- and thyrotropin-releasing hormone parvocellular neurons that inhibit feeding (Kola et al., 2008). Kola and colleagues have demonstrated that administration of compounds that inhibit endocannabinoid synthesis, such as calcium chelators and tetrahydrolipstatin (inhibits 2-AG synthesis), directly into these PVN parvocellular neurons circumvents ghrelin-induced inhibition of miniature excitatory postsynaptic currents (mEPSC) onto these satiety neurons (Gao and Horvath, 2007; Kola et al., 2008). Together, these

experiments highlight that ghrelin stimulates the production and release of 2-AG from parvocellular neurons, which retrogradely inhibits glutamate release onto satiety promoting PVN parvocellular neurons (Kola et al., 2008). This discovery complements earlier work, whereby leptin was shown to reduce endocannabinoid-mediated inhibition onto both PVN parvocellular and magnocellular neurons (Di et al., 2003, 2005; Malcher-Lopes et al., 2006). Although this is one example of where ghrelin and endocannabinoid systems interact within the HYP to modulate feeding circuits, more research will undoubtedly uncover additional hypothalamic neurocircuits where these systems influence one another.

1.4 Ghrelin and endocannabinoid systems involved in non-homeostatic reward-based feeding

It is now well recognized that feeding is not solely regulated by hunger driven homeostatic mechanisms within the HYP; higher order non-homeostatic mechanisms, which integrate rewarding properties of food with learned experiences, likewise contribute by initiating appropriate motivated behaviours to obtain and consume desired foods in the absence of hunger (Liu and Kanoski, 2018). In addition to playing an instrumental role in promoting homeostatic feeding within the HYP, ghrelin and endocannabinoid systems are now known for the integral role they play in regulating non-homeostatic reward-based motivated feeding behaviours (Edwards and Abizaid, 2016).

1.4.1 Ghrelin and endocannabinoid systems increase the reward associated with and motivation to obtain food

Modulation of the ghrelin system alters the motivational and rewarding aspects associated with obtaining and consuming foods. Accordingly, ghrelin administration facilitates the development of conditioned place preferences (CPPs) (i.e. increases how rewarding and motivating) and enhances operant breakpoints (i.e. increases the effort that rodents are willing

to exert) for food rewards (Davis et al., 2007; Jerlhag, 2008; Perello et al., 2010; Skibicka et al., 2011). Importantly, peripheral and central administration of ghrelin increases the effort rodents are willing to expend to obtain food rewards even when rodents are satiated (Skibicka et al., 2012). This demonstrates that ghrelin can engage motivational processes to drive feeding behaviours in the absence of hunger (i.e. non-homeostatic feeding) (Skibicka et al., 2012). Moreover, although levels of circulating ghrelin rise in anticipation of most meals, this rise tends to be more pronounced when individuals consider the meal rewarding, which suggests that ghrelin is likewise involved in the hedonic aspect of feeding (Monteleone et al., 2012). Conversely, pharmacological (GHSR antagonist treated) or genetic (GHSR KO) disruption of ghrelin signaling prevents the development of CPP and decreases breakpoints for palatable foods suggesting that GHSR signaling is essential for the attractiveness and desire to obtain food rewards (Jerlhag et al., 2009; Egecioglu et al., 2010; Jerlhag et al., 2010; Perello et al., 2010; Jerlhag et al., 2011a; Jerlhag and Engel, 2011; Skibicka et al., 2011). Further strengthening the argument that ghrelin acts via GHSRs to enhance non-homeostatic feeding, peripheral administration of ghrelin increases the intake of non-caloric sweet tasting food and drinks (flavoured saccharin) in WT but not in GHSR deficient mice (Disse et al., 2010). Together, these studies confirm the integral role that the ghrelin system plays in promoting the desirability of foods and subsequent motivational behaviours required to obtain and consume them.

Similarly, modulation of endocannabinoid signaling also influences how motivated animals are to acquire and consume foods as well as the hedonic impact that these foods convey. Accordingly, CB-1R agonists increase, whereas CB-1R antagonists decrease the effort rodents are willing to exert to obtain palatable food rewards (Perio et al., 2001; Solinas and Goldberg, 2005). Consistent with this, CB-1R-KO mice are also inherently less motivated to bar press for sucrose solutions and reduce their consumption of sucrose compared to WTs (Arnone et al., 1997; Simiand et al., 1998; Poncelet et al., 2003; Sanchis-Segura et al., 2004). Interestingly, pretreatment of rodents with CB-1R antagonists sufficiently prevents CPPs associated with classical reinforcers (e.g. cocaine, morphine, food) suggesting that the endocannabinoid system regulates the perception of rewarding stimuli in general (Chaperon et

al., 1998). The endocannabinoid system also plays an important role in hedonic feeding as CB-1R agonists decrease the rejection of adverse solutions, increase the positive ingestive responses to rewarding solutions, and promote feeding in satiated animals (Werner and Koch, 2003; Jarrett et al., 2005; Solinas and Goldberg, 2005; Jarrett et al., 2007; Sinnayah et al., 2008). Conversely, CB-1R antagonists increase the rejection of adverse solutions, decrease palatability of rewarding solutions and blunt re-feeding responses in food deprived rodents (Werner and Koch, 2003; Jarrett et al., 2005; Solinas and Goldberg, 2005; Jarrett et al., 2007; Sinnayah et al., 2008). Human studies also suggest that endocannabinoid signaling is important for the manifestation of hedonic feeding behaviours. Accordingly, people describe heightened and reduced food cravings upon endocannabinoid system stimulation and antagonism, respectively (Kirkham, 2009). Consistent with this, the elevation of circulating 2-AG that accompanies food presentation is more pronounced when foods are anticipated to be palatable or rewarding compared to those observed when non-palatable foods are presented (Monteleone et al., 2012). Furthermore, although 2-AG levels drop following meal initiation, 2-AG levels remain higher in individuals consuming their favourite (i.e. rewarding) food relative to levels observed in calorically matched but less palatable food (Monteleone et al., 2016). Collectively, these experiments highlight that CB-1R signaling is important for the manifestation of hedonically driven motivated feeding behaviours.

1.4.2 Mesocorticolimbic dopamine reward system engagement

The mesocorticolimbic dopamine system has long been recognized as the “master regulator of motivated and reward seeking behaviours” (Berridge, 2009; Volkow et al., 2011). This system consists of dopaminergic neurons that originate in the VTA and their corresponding neural projections to corticolimbic structures such as the NA, prefrontal cortex (PFC), hippocampus (HIP), and the amygdala (refer to Supplementary figure 1) (Berridge and Robinson, 2003; Alcaro et al., 2007; Björklund and Dunnett, 2007). Augmented dopamine transmission within these target regions, induced by heightened VTA dopamine neuron activity, occurs in response to and in anticipation of extremely rewarding stimuli, such as drugs of abuse but also occurs in response to natural rewards such as foods (Richardson and Gratton, 1996;

Lutter and Nestler, 2009). Increased dopamine release within the mesocorticolimbic system, particularly the NA, is thought to encourage and coordinate a variety of processes such as increased arousal, psychomotor recruitment, and reward related conditioned learning to engage motivated behaviours to obtain and consume rewarding stimuli (Lutter and Nestler, 2009). Appropriately, this system plays an instrumental role in coordinating motivated non-homeostatic feeding behaviours and is sensitive to and regulated by many important feeding signals, including ghrelin and endocannabinoids (Liu and Borgland, 2015).

Consistent with the role of ghrelin in promoting non-homeostatic motivated feeding, functional magnetic resonance imaging (fMRI) studies detect increased engagement of the mesocorticolimbic dopamine system in food related paradigms when the ghrelin system is artificially or naturally promoted (Malik et al., 2008; Wellman et al., 2011; Goldstone et al., 2014). Accordingly, fMRI studies detect increased activity in mesolimbic dopaminergic circuits following intravenous ghrelin injections in mice (Wellman et al., 2011). Moreover, ghrelin is also thought to engage this system naturally as although palatable food rewards enhance NA dopamine levels in WT mice, this effect is absent in GHSR KO mice (Egecioglu et al., 2010). Intriguingly, similar processes and brain regions are also engaged in humans, as high ghrelin levels, increased either endogenously (i.e. by fasting) or by external administration, enhances food appeal and hunger ratings in humans and is associated with heightened mesocorticolimbic neural responses and blood oxygen-level dependent signals (Malik et al., 2008; Goldstone et al., 2014). Together, these studies suggest that in food related contexts, ghrelin plays a facilitative role in engaging the mesocorticolimbic system.

Given the endocannabinoid systems role in modulating non-homeostatic motivated feeding behaviours, it is not surprising that it likewise engages the mesocorticolimbic dopamine system to influence non-homeostatic appetite. In support of this, exogenous (e.g. THC administration) and endogenous (e.g. genetic mutations that heighten endocannabinoid levels) CB-1R agonist augmentation is evidenced to stimulate dopamine release, heighten reward related ventral striatum reactivity, and increase palatable food consumption (Foltin et al., 1988;

Hariri et al., 2009; Bossong et al., 2015). Moreover, although fMRI studies traditionally demonstrate strong activation of mesocorticolimbic brain regions upon palatable food reward presentation, this activation is attenuated when CB-1Rs are antagonized (Horder et al., 2010). Fittingly, the natural augmentation of NA dopamine that is induced by the presentation of novel palatable foods is also prevented by CB-1R antagonism (i.e. rimonabant pretreatment) (Melis et al., 2007). Collectively, these studies demonstrate that endocannabinoids act within the mesocorticolimbic dopamine system to modulate non-homeostatic appetite and feeding.

1.4.3 The VTA: hub and master regulator of the mesocorticolimbic dopamine system

The VTA is largely considered to be the hub of the mesocorticolimbic dopamine system as it houses the dopaminergic neurons that coordinate mesocorticolimbic dopamine neurotransmission and subsequent motivated behaviours (Melis and Pistis, 2012). The VTA is most renowned for housing the mesocorticolimbic dopamine neurons, which represent 50–65% of VTA neurons; however, GABA (30–35%) and glutamate (5–45%) neurons also reside within this ventromedial midbrain region (Yamaguchi et al., 2007, 2011; Nair-Roberts et al., 2008; Li et al., 2013). The wide discrepancy in the relative proportions of these three cell types has been driven by differences in immunohistochemical and *in situ* hybridization procedures but also by misclassifying these neurons as distinctly one of these three cell types (Margolis et al., 2006; Nair-Roberts et al., 2008; Li et al., 2013; Root et al., 2014; Zhang et al., 2015; Berrios et al., 2016; Morales and Margolis, 2017; Root et al., 2018). Although new roles and behaviours are beginning to be attributed to the selective activation of non-dopaminergic neurons within the VTA, the dopaminergic neurons remain the most commonly studied output neurons of the VTA with respect to reward and motivated behaviours (Tan et al., 2012; van Zessen et al., 2012; Stamatakis et al., 2013; Wang et al., 2015a; Berrios et al., 2016; Qi et al., 2016; Morales and Margolis, 2017). Interestingly, the non-dopaminergic neurons of the VTA project to and help regulate the excitability of these primary VTA dopaminergic neurons (Dobi et al., 2010; Tan et al., 2012). Accordingly, these dopaminergic neurons receive strong GABAergic axon collateral inputs from local VTA GABAergic neurons as well as excitatory inputs from VTA glutamatergic

neurons (Omelchenko and Sesack, 2009; Dobi et al., 2010). In addition to modulating the activity of dopaminergic neurons directly, these VTA glutamatergic neurons also innervate neighbouring GABAergic interneurons (Dobi et al., 2010). Therefore these neurons also have the capacity to indirectly influence the excitability of dopaminergic VTA neurons by modulating the activity of GABAergic interneurons (Dobi et al., 2010). VTA dopaminergic neurons are also strongly innervated by external glutamatergic excitatory inputs of the PFC and bed nucleus of the stria terminalis (BNST), glutamatergic/cholinergic excitatory neurons of the pedunculo pontine (PPT) and laterodorsal tegmental nuclei (LDT), and GABAergic inhibitory afferents of the ventral striatum, ventral pallidum (VP), and the rostromedial tegmental nucleus (RMTg) (depicted in Supplementary figure 1) (Omelchenko and Sesack, 2007; Morikawa and Paladini, 2011). The extensive innervation of VTA dopamine neurons by excitatory and inhibitory inputs (local and external sources) and evidence that these inputs can functionally influence their activity and firing rate suggest that VTA dopamine neurons are ultimately controlled by an intricate balance of excitatory and inhibitory tone onto them (Melis and Pistis, 2012).

1.4.4 Ghrelin and endocannabinoid systems act within the VTA to drive motivated non-homeostatic reward-based feeding

Given the aforementioned involvement that ghrelin and endocannabinoid systems have in engaging the mesocorticolimbic system to promote motivated feeding behaviours and that the VTA is the central node of this system, it is fitting that the VTA has receptors for and is sensitive to both ghrelin and endocannabinoids (Herkenham et al., 1991; Guan et al., 1997; Abizaid et al., 2006b; Mátyás et al., 2008; Mani et al., 2014; Liu and Borgland, 2015; Edwards and Abizaid, 2016).

In situ hybridization and immunohistochemical studies demonstrate that a sizable proportion of VTA dopaminergic (40–60%) and GABAergic neurons (~30%) express GHSRs (Abizaid et al., 2006b; Zigman et al., 2006). Consistent with the expression of GHSRs in dopaminergic neurons of the VTA and the excitatory nature of G-protein-coupled GHSRs ($G\alpha_q$),

ghrelin increases the neuronal excitability of VTA dopaminergic neurons, augments dopamine release and turnover within the NA, and potentially stimulates food intake and motivated feeding behaviours when it is infused into the VTA (Guan et al., 1997; Abizaid et al., 2006b; Zigman et al., 2006; Jerlhag et al., 2007; King et al., 2011; Cone et al., 2014; Mani et al., 2014; van der Plasse et al., 2015). Importantly, the ability of ghrelin to enhance the neuronal excitability of VTA dopaminergic neurons, augment dopamine turnover in the NA, and induce food intake are GHSR-dependent as these effects are abolished in GHSR-KO rodents (Abizaid et al., 2006b). In agreement with this, chronic intra-VTA administration of ghrelin enhances; whereas, GHSR antagonists decrease both the consumption and the effort rodents are willing to expend (i.e. motivation) to obtain palatable foods in progressive ratio experiments (King et al., 2011; Weinberg et al., 2011). Moreover, intra-VTA administration of a GHSR antagonist is sufficient to prevent the orexigenic effect of endogenously high (elevated by 24-hour fast) and peripherally injected ghrelin (at a physiologically relevant dose) suggesting that peripheral ghrelin can target the VTA to stimulate GHSR-mediated feeding (Abizaid et al., 2006b). It is important to note that GHSRs also have extremely high constitutive activity (Holst et al., 2003; Holst and Schwartz, 2004); therefore, it is possible that the lack of feeding effects in response to the combined administration of IP ghrelin and intra-VTA GHSR antagonist could also be reflective of reduced GHSR constitutive activity within the VTA (Abizaid et al., 2006b). In addition to being dependent on GHSRs, the ability of ghrelin to induce motivated feeding behaviours relies on striatal dopamine as rodents that are infused (intra-VTA) with the dopaminergic neuron neurotoxin, 6-hydroxydopamine, are less motivated to search and bar press for palatable foods following central ghrelin infusions compared to controls (Weinberg et al., 2011). Furthermore, unlike GHSR deficient rodents, who do not develop a CPP for palatable food rewards or increase their food intake after ghrelin administration, mice that only express GHSRs in tyrosine hydroxylase (TH)-positive neurons demonstrate CPP for rewarding foods and increase their food intake in response to peripheral ghrelin treatment (Chuang et al., 2011). Together, these studies highlight that ghrelin-induced activation of GHSRs within the VTA increases the neuronal excitability of mesocorticolimbic dopaminergic neurons, enhances dopamine release and turnover within the NA, and stimulates motivated feeding behaviours.

Ghrelin stimulates VTA dopaminergic neurons directly through the activation of excitatory G-protein-coupled GHSRs on their surface and indirectly by promoting excitatory and reducing inhibitory afferents that innervate these neurons (Supplementary figure 2, Panel A) (Abizaid et al., 2006b). Accordingly, bath application of ghrelin increases the frequency of action potentials in VTA dopaminergic neurons and peripheral administration of ghrelin reduces inhibitory but increases excitatory inputs (number of symmetric versus asymmetric synapses) and tone (mIPSCs and mEPSCs) onto dopaminergic neurons of the VTA (Abizaid et al., 2006b). Importantly, ghrelin requires excitatory synaptic transmission to significantly increase the frequency of action potentials in VTA dopaminergic neurons as its ability to do so is severely hindered by co-application of glutamate receptor antagonists (e.g. cyanquinoxaline (CNQX) and 2-amino-5-phosphopentanoic acid (AP-5)) (Abizaid et al., 2006b). Furthermore, these processes are GHSR dependent as ghrelin does not increase action potential firing of VTA dopaminergic neurons nor does it induce the synaptic remodelling that enhances the probability of their activation in GHSR KOs (Abizaid et al., 2006b). In support of the presynaptic and postsynaptic actions of ghrelin within the VTA, ghrelin-binding and GHSR-immunoreactivity studies demonstrate immunolabelling patterns that are consistent with both a presynaptic and postsynaptic localization of GHSRs (Abizaid et al., 2006b). Ghrelin-mediated activation of these VTA dopaminergic neurons increases the concentration of extracellular dopamine and dopamine turnover in the NA and consequentially engages and enhances motivational processes to find and consume foods (Abizaid et al., 2006b; Jerlhag et al., 2007; King et al., 2011; Cone et al., 2014; van der Plasse et al., 2015).

Given that the endocannabinoid system activates the mesocorticolimbic dopamine system and is able to influence non-homeostatic feeding behaviours, it is not surprising that CB-1Rs and the enzymes needed for the synthesis and degradation of endocannabinoids are expressed within the VTA (Matsuda et al., 1990, 1993; Herkenham et al., 1991; Kirkham et al., 2002; Mackie, 2005; Mátyás et al., 2008). Similar to ghrelin administration, *in vitro* and *in vivo* application of CB-1R agonists stimulates VTA dopaminergic neuron activity and burst firing, heightens dopamine release within the NA shell, and potently increases food intake and

motivated feeding behaviours (Chen et al., 1990; French, 1997; Gifford et al., 1997; Tanda et al., 1997; Gessa et al., 1998; Cheer et al., 2000; Solinas et al., 2006; Sinnayah et al., 2008; Oleson et al., 2012; Wang et al., 2015b). These effects are mediated by CB-1Rs as they are blocked following CB-1R antagonist pretreatment (Tanda et al., 1997; Gessa et al., 1998; Sinnayah et al., 2008; Oleson et al., 2012). Importantly, intra-VTA injections of CB-1R antagonists, in the absence of exogenous administration of CB-1R agonists, also reduces cue-evoked dopamine concentrations within the NA, food intake, and food seeking behaviours (Sinnayah et al., 2008; Oleson et al., 2012). This suggests that tonic endocannabinoid tone is important for regulating these processes within the VTA. Interestingly, augmenting 2-AG levels within the VTA by inhibiting the enzyme (i.e. MAGL) that naturally degrades 2-AG is also sufficient to stimulate dopaminergic neurons of the VTA, induce increases in dopamine release within the NA, and promote motivated behaviours to obtain and consume foods (Oleson et al., 2012; Wang et al., 2015b). Collectively, these experiments demonstrate that like ghrelin-induced activation of GHSRs, CB-1R-mediated endocannabinoid signaling within the VTA is important for stimulating dopaminergic neurons that promote motivated non-homeostatic feeding behaviours.

CB-1R mRNA and protein are expressed within the VTA (Herkenham et al., 1991; Matsuda et al., 1993; Mátyás et al., 2008; Merrill et al., 2015; Friend et al., 2017; Han et al., 2017). Despite a few immunohistochemical studies reporting weak co-localization of CB-1Rs in TH-expressing cells of the VTA (Hernandez et al., 2000; Wenger et al., 2003), a majority of studies suggest that CB-1Rs are not expressed directly in dopamine neurons but rather in GABAergic and glutamatergic neurons of the VTA (Herkenham et al., 1991; Mackie, 2005; Mátyás et al., 2008; Fitzgerald et al., 2012; Han et al., 2017). Consistent with the retrograde actions of endocannabinoids, CB-1Rs are predominately expressed on the axon terminals of these local non-dopaminergic VTA neurons. Interestingly, GABAergic and glutamatergic CB-1R-positive axon terminals from these local non-dopaminergic VTA neurons synapse onto dopaminergic neurons of the VTA (Mátyás et al., 2008). In addition to these inputs, VTA dopaminergic neurons also receive dense innervations from CB-1R-positive GABAergic and

glutamatergic afferents from extrinsic brain regions (e.g. PFC, RMTg) (Szabo et al., 2002; Melis et al., 2004a, 2004b; Mátyás et al., 2008).

DAGL- α , the enzyme that synthesizes 2-AG, is ubiquitously expressed in both dopaminergic and non-dopaminergic neurons of the VTA (Mátyás et al., 2008). High-resolution electron microscopy demonstrates that DAGL- α immunostaining is concentrated perisynaptically in dendrites of postsynaptic neurons specifically apposed to CB-1R-positive axon terminals forming both symmetric (putative GABAergic) and asymmetric (putative glutamatergic) synapses (Mátyás et al., 2008). In contrast, NAPE-PLD, the enzyme that primarily synthesizes AEA is negligibly expressed within the VTA (Egertova et al., 2008). Consistent with the high and low expression of DAGL- α and NAPE-PLD, respectively, 2-AG levels are reported to be approximately 100 to 1000 fold those of AEA within the midbrain (Bisogno et al., 1999; Egertova et al., 2008; Mátyás et al., 2008). Appropriately, 2-AG is largely considered to be the endocannabinoid responsible for stimulating dopaminergic neurons of the VTA and for inducing associated motivated feeding behaviours (Oleson et al., 2012; Wang et al., 2015b). Collectively, these data support a model whereby dopaminergic neurons of the VTA regulate GABA and glutamate release onto them via 2-AG CB-1R-mediated inhibition of neurotransmitter release.

This model is supported by a substantial number of electrophysiology studies demonstrating reduced GABA and glutamate neurotransmission onto VTA dopaminergic neurons following bath application of CB-1R agonists (Szabo et al., 2002; Melis et al., 2004a, 2004b; Riegel and Lurpica, 2004; Mátyás et al., 2008; Kortleven et al., 2011; Labouebe et al., 2013; Wang et al., 2015b). Accordingly, CB-1R agonists suppress GABA_A and GABA_B receptor-mediated eIPSCs in VTA dopaminergic neurons (Szabo et al., 2002; Riegel and Lurpica, 2004; Wang et al., 2015b). Conversely, application of CB-1R antagonists increase GABA_B receptor-mediated eIPSCs indicating that endocannabinoids tonically inhibit these GABA_B eIPSCs in dopaminergic neurons of the VTA (Wang et al., 2015b). In support of the aforementioned localization of DAGL- α expression in VTA dopamine neurons, 2-AG is the endocannabinoid responsible for this tonic inhibition as suppression of 2-AG biosynthesis blocks the increase in

GABA_B-mediated eIPSCs induced by CB-1R agonism (Mátyás et al., 2008; Wang et al., 2015b). Along the same lines, endocannabinoids and CB-1R agonists demonstrate the capacity to presynaptically inhibit glutamate transmission onto dopaminergic neurons of the VTA (Melis et al., 2004b, 2004a). Accordingly, CB-1R agonists reduce N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated evoked excitatory postsynaptic currents (eEPSCs) (Melis et al., 2004a, 2004b). Inhibition of glutamate release from extrinsic glutamatergic afferents is presumed to be the neural substrate of the reduction in VTA dopaminergic neuron EPSCs following CB-1R agonist treatment as these afferents strongly project to and regulate the activity of VTA dopamine neurons (Melis et al., 2004a). The suppression of glutamate and GABA release from excitatory and inhibitory afferents is specifically mediated by CB-1Rs as CB-1R antagonists attenuate cannabinoid-induced inhibition of eEPSCs and eIPSCs, respectively (Szabo et al., 2002; Melis et al., 2004a, 2004b; Riegel and Lurpica, 2004).

The way in which the endocannabinoid system stimulates dopaminergic neuron activity within the VTA remains incompletely understood. Nevertheless, given that CB-1R agonists increase VTA dopaminergic neuron activity and dopamine release within the NA, it is hypothesized that while endocannabinoids may inhibit both glutamate and GABA release onto dopaminergic neurons of the VTA, endocannabinoids likely suppress GABA inhibitory tone more effectively (Supplementary figure 2, Panel B) (Oleson and Cheer, 2012; Edwards and Abizaid, 2016). Theoretically, this could be due to a larger quantity of innate CB-1R-positive inhibitory synapses relative to excitatory synapses contacting VTA dopamine neurons. Alternatively, endocannabinoid signaling machinery may be more strongly or preferentially expressed at inhibitory versus excitatory synapses (a phenomenon observed in other brain regions) (Uchigashima et al., 2007; Yoshida et al., 2011). In support of the former, GABAergic neurons are the second most abundant cell type in the VTA (aside from dopaminergic neurons) and these neurons strongly innervate and regulate the activity of VTA dopamine neurons (with the help of extra-VTA GABAergic inputs) (Bayer and Pickel, 1991; Johnson and North, 1992; Grace et al., 2007; Omelchenko and Sesack, 2009; Bourdy and Barrot, 2012). Consistent with this,

Abizaid and colleagues (2006) report higher frequencies of miniature IPSCs (mIPSCs) compared to miniature EPSCs (mEPSCs) and more putative GABAergic relative to glutamatergic synapses at VTA dopaminergic neurons in vehicle treated animals. Additionally, pharmacology experiments have suggested that although dopaminergic neurons of the VTA have receptors for and are responsive to GABA, glutamate, and acetylcholine, the tonic regulation of VTA dopaminergic neurons is predominately mediated by GABA_A receptors (Westerink et al., 1996; Parker et al., 2011). Further supporting the notion that endocannabinoids activate VTA dopaminergic neurons by suppressing inhibitory tone onto them, bicuculline (GABA_A receptor antagonist) pretreatment occludes increases in VTA dopaminergic activity traditionally observed with CB-1R agonist administration (Cheer et al., 2000). Appropriately, removal of GABA inhibitory tone has been shown to induce burst firing in dopaminergic neurons of the midbrain (Lobb et al., 2010). Collectively, these experiments suggest that CB-1R agonists dampen tonic GABA neurotransmission onto the dopaminergic neurons of the VTA to increase their activity.

In support of this, the RMTg, a brain region more colloquially referred to as the tail of the VTA, contains a dense population of CB-1R-positive GABAergic neurons that strongly innervate and modulate the activity of a large proportion (60–95%) of dopaminergic neurons within the VTA (Kaufling et al., 2010; Balcita-Pedicino et al., 2011; Hong et al., 2011; Lecca et al., 2011, 2012; Bourdy and Barrot, 2012). Electrical stimulation of the RMTg potently inhibits dopaminergic neurons of the VTA (Lecca et al., 2011). Intriguingly, CB-1R agonists potently inhibit RMTg-induced suppression of VTA dopaminergic neurons by reducing GABA release from these neurons onto VTA dopaminergic neurons (Lecca et al., 2012). Suitably, these RMTg inhibitory projections express presynaptic CB-1Rs and exhibit reduced GABA release following VTA dopaminergic neuron stimulation (a process known to promote 2-AG synthesis) (Lecca et al., 2011, 2012; Melis et al., 2014). Importantly, the reduction in GABA release from putative RMTg following stimulation of VTA dopaminergic neurons is attenuated upon bath application of CB-1R antagonists and when DAGL- α inhibitors are infused into dopaminergic neurons of the VTA (Melis et al., 2014). This demonstrates that dopaminergic neurons of the VTA can disinhibit

their own activity via 2-AG-mediated activation of presynaptic CB-1Rs on GABAergic terminals innervating them.

1.5 Overall objectives and hypotheses

Ghrelin and endocannabinoid systems have overlapping receptor localization, similar expression profiles of their orexigenic ligands, and a shared capacity to promote adiposity and food intake. These similarities led to postulations that ghrelin and endocannabinoid systems may collaboratively regulate these processes in peripheral organs associated with metabolism (i.e. stomach, adipose tissue, and liver) and brain regions known to regulate feeding (e.g. HYP). It has become clear that this is indeed the case as disruption of either GHSR or CB-1R signaling eliminates the traditional ability of either ghrelin or endocannabinoids to promote adipogenic processes in the periphery and their capacity to initiate orexigenic pathways and induce homeostatic feeding behaviours within the HYP.

Interestingly, GHSRs and CB-1Rs are likewise expressed in the VTA, a brain region essential for driving reward and motivated feeding behaviours. Within this region, independent activation of either GHSRs or CB-1Rs stimulates dopaminergic neurons, enhances dopamine release within mesocorticolimbic targets, and consequentially promotes non-homeostatic feeding. Given evidence of their interaction elsewhere and the functional similarities between these systems within the VTA, **we predict that ghrelin and endocannabinoid systems interact within the VTA and that this interaction is essential for encouraging non-homeostatic motivated feeding behaviours.**

Previous work from our lab supports a putative interaction between ghrelin and endocannabinoid systems within the VTA. We have demonstrated that VTA CB-1R mRNA expression is reduced in rats with disturbed GHSR signaling (i.e. GHSR KO) relative to WTs suggesting that GHSR signaling influences CB-1R mediated signaling within the VTA. We have

also demonstrated that peripheral CB-1R antagonism attenuates the capacity of ghrelin to stimulate food intake when it is infused into the VTA. This indicates that a functional endocannabinoid system is likewise required for the orexigenic effect of intra-VTA ghrelin. The present thesis aims to build on these findings by confirming that ghrelin and endocannabinoid systems interact within the VTA (Chapter 2), assessing whether intra-VTA endocannabinoid signaling is required for ghrelin driven food intake and motivated feeding behaviours (Chapter 3), and dissecting the neural mechanisms underlying this interaction (Chapter 4).

Chapter 2:

Examining if ghrelin and endocannabinoid systems interact within the VTA

2.1 Rationale and overall approach

It is clear that ghrelin and endocannabinoid systems interact with one another within the periphery and within the HYP to promote adipogenic and orexigenic processes (Kirkham et al., 2002; Kola et al., 2005; Kola, 2008; Zbucki et al., 2008; Kola and Korbonits, 2009; Kola et al., 2013; Lim et al., 2013). Accordingly, disruption of either GHSRs or CB-1Rs eliminates the adipogenic and orexigenic capacity of both ghrelin and endocannabinoids (Cani et al., 2004; Kola et al., 2005, 2013; Kola, 2008; Kola and Korbonits, 2009; Lim et al., 2013; Senin et al., 2013). We predicted that these systems also interact within the VTA given evidence of their interaction elsewhere, the joint expression of their receptors within the VTA, and the overall similarity in behaviours that accompany intra-VTA infusion of ghrelin or endocannabinoids. We reasoned that if these systems interact within the VTA then disruption of one system should induce detectable alterations in the other within this region. Consistent with this, we previously demonstrated that ghrelin receptor deficient (GHSR KO) rats have decreased gene expression of CB-1Rs within the VTA (Edwards, 2014). Although these data support a putative interaction between ghrelin and endocannabinoid systems within the VTA, we herein sought to further characterize and corroborate the existence of this interaction within the VTA. To this end, we compared VTA endocannabinoid concentrations and the gene expression of endocannabinoid system proteins involved in the synthesis and degradation of these endocannabinoids in GHSR KO and WT rats. Liquid chromatography-mass spectroscopy (LC-MS) and reverse transcription quantitative polymerase chain reaction (RT-qPCR) methodologies were used to quantify endocannabinoid concentrations and to examine gene expression differences between genotypes, respectively.

2.2 General methods

2.2.1 Animals

All procedures described were approved by the Carleton University Animal Care Committee and strictly followed the guidelines laid out by the Canadian Council on Animal Care. The ghrelin receptor deficient rats, referred to herein as GHSR KOs, are Fawn Hooded Hypertensive rats (*FHH-GHSR^{m1/Mcwi}*) that have a truncated receptor due to a point mutation in their GHSR gene. These rats do not demonstrate classical ghrelin-mediated responses following ghrelin injection and have deficiencies in GHSR-mediated behaviours (Clifford et al., 2012; MacKay et al., 2016; Hyland et al., 2018). Male GHSR KO and WT (Fawn Hooded Hypertensive-WT) littermates (originally obtained from Transposagen Biopharmaceuticals Incorporate) were bred in-house at Carleton University for both LC-MS and RT-qPCR endocannabinoid quantification experiments. Rats were pair-housed in plexiglass cages (48 cm x 26 cm x 20 cm) after weaning in temperature (22°C) and humidity (45 to 55%) controlled rooms with *ad libitum* access to chow (Harlan Diets; 3.3 kcal/g; 4% fat; 48% carbohydrates; 14.3% protein) and water. Rats (aged three to four months) were sacrificed during their light cycle (between 08:00 and 12:00).

2.2.2 Tissue processing

For endocannabinoid quantification experiments, rats were sacrificed solely by rapid decapitation to minimize distortion of physiological endocannabinoid concentrations that are known to spike during stress (e.g. CO₂ asphyxiation) and shortly after death (Buczynski and Parsons, 2010). For gene expression studies, rats were sacrificed via CO₂ asphyxiation followed by rapid decapitation. Brains were immediately extracted from the skull following decapitation and flash frozen on dry ice. A 1 mm coronal brain slice, containing the VTA, was cut and the VTA was microdissected out with the aid of a dissection microscope. VTA micropunches were

transferred to Eppendorf tubes and stored at -80°C until required for LC-MS or RT-qPCR experiments.

2.2.3 2-AG and AEA extraction and quantification via liquid chromatography-mass spectroscopy

Pre-weighed frozen VTA micropunches from GHSR KO and WT rats were sent to the lab of Dr. Matthew Hill (Hotchkiss Brain Institute, Calgary, Alberta, Canada) for endocannabinoid extraction and quantification via LC-MS. VTA micropunches were subjected to a lipid extraction process as described previously (Patel et al., 2003; Bowles et al., 2012). Briefly, samples were homogenized with a glass rod in borosilicate glass tubes containing 2 ml of acetonitrile, 186 pmol of $[^2\text{H}_8]$ 2-AG, and 84 pmol of $[^2\text{H}_8]$ anandamide. Samples were sonicated for 30 minutes, incubated at -20°C overnight to precipitate proteins, spun at $1500 \times g$, and supernatants were transferred to new glass tubes. Samples were dried under N_2 , rinsed with methanol to minimize lipid loss, and dried again under N_2 . 20 μl of methanol was used to resuspend lipid extracts containing the endocannabinoids. 2-AG and AEA levels were subsequently separated, identified, and quantified via isotope-dilution LC-MS as described in detail in (Patel et al., 2005).

2.2.4 RT-qPCR endocannabinoid system gene expression analysis

VTA micropunches were homogenized in TriZol reagent (Invitrogen, Carlsbad, CA) to encourage release of cell contents and dissociation of nucleoprotein complexes. Chloroform was added to each sample prior to centrifugation (at $12\,000 \times g$ for 15 minutes) to separate the RNA aqueous phase from the DNA and protein containing organic phases. Isopropyl alcohol and linear acrylamide were added to each sample to facilitate RNA precipitation from the aqueous phase upon centrifugation (at $12\,000 \times g$ for 15 minutes). RNA pellets were washed in 75% ethanol and dissolved in RNase free water. The extracted RNA was kept at -80°C until required for reverse transcription complementary DNA (cDNA) synthesis.

On the day of cDNA synthesis, the concentration and quality of extracted RNA samples were assessed using a Thermo Scientific Nanodrop 100 spectrophotometer (Waltham, MA). Only RNA of sufficient quality and concentration were reversed transcribed into cDNA using a SuperScript II Reverse Transcriptase (SSII RT) kit and the method provided by the manufacturer (Life Technologies, Carlsbad, CA). Briefly, oligo (dT) primers (Invitrogen) were mixed with diluted RNA samples (5000 ng of RNA per sample) and then heated for 5 minutes at 70°C. An aliquot of master mix containing 5 X first strand buffer (Invitrogen), dithiothreitol (Invitrogen), RNase inhibitor (Promega, Madison, WI), deoxynucleotide triphosphate (Invitrogen), DEPC water and SSII RT (Invitrogen) was added to each sample as specified by the manufacturer (Life Technologies). Samples were run in a MJ Research PTC-200 Thermal Cycler (Marshall Scientific, Hampton, NH) at 42°C for 1.5 hours. A final 10-minute 90 °C cycle was used to inactivate the reverse transcriptase. cDNA samples were stored at –20 °C until required for RT-qPCR experiments.

The nucleotide sequences of all qPCR primers used are included in Appendix A (Supplemental table 1). All primer pairs were tested for their amplification efficiencies using a standard curve. Only primer pairs which fell between 90 and 110% efficient were utilized for RT-qPCR experiments. Primers, cDNA (diluted to a concentration within the linear dynamic range of the primer pair standard curve), MilliQ H₂O, and iQ SYBR Green PCR Super Mix were combined according to the method of the manufacturer (BioRad, Hercules, CA). All samples including non-template and non-reverse transcription controls were run in triplicates using primers for the gene of interest as well as corresponding *GAPDH* and *Actb* housekeeping genes. PCR plates were run on a CFX Connect™ Real-Time PCR machine (BioRad) using the following program (with slight modification to the annealing temperature depending on the melting temperature of primers): 30 second 95°C step, 40 cycles of denaturing (10 seconds at 95°C), annealing (30 seconds at ~55°C), and extension steps (20 seconds at 72°C), and a final one-minute 95°C step. RT-qPCR data was analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.2.5 Data analyses

The effect of genotype on endocannabinoid content was assessed using independent samples t-tests with genotype (GHSR KO vs WT) as the independent grouping variable and VTA concentration (2-AG or AEA) as the dependent variable. Similarly, $2^{-\Delta\Delta C_t}$ values computed following RT-qPCR experiments, were analyzed via independent samples t-tests with genotype (GHSR KO vs WT) as the independent grouping variable and $2^{-\Delta\Delta C_t}$ values assigned as the dependent test variable. A probability value of $p < 0.05$ was used to assess statistical significance for all conducted t-tests. All statistics were conducted with IBM SPSS 21 statistical software. Results were represented as the mean $\pm$ S.E.M.

2.3 Results

2.3.1 Genetic disruption of GHSR signaling decreases VTA 2-AG levels

To examine if genetic disruption of GHSR signaling modulates the concentration of 2-AG and AEA within the VTA, we compared endocannabinoid levels within VTA micropunches from GHSR KO ($n = 10$) and WT ($n = 10$) rats. LC-MS separation and quantification of endocannabinoids, revealed that GHSR KOs had significantly lower VTA 2-AG concentrations ($t_{(18)} = 2.172$, $p = 0.043$, $d = 0.971$) compared to WT rats (Figure 2.1). In contrast, VTA AEA concentrations ($t_{(18)} = 1.276$, $p = 0.218$, $d = 0.571$) did not significantly differ between genotypes.

Figure 2.1: Genetic deletion of functional GHSRs reduces 2-AG levels in the VTA. Comparison of WT and GHSR KO **(A)** 2-AG and **(B)** AEA concentrations within the VTA. * $p < .05$.

2.3.2 Genetic disruption of GHSR signaling suppresses the gene expression of endocannabinoid system proteins within the VTA

We compared the gene expression of enzymes that synthesize and break down 2-AG and AEA in GHSR KO (n = 11–12) relative to WT (n= 6–7) rats (Figure 2.2) to examine if they may help explain the altered endocannabinoid levels found in GHSR KOs. Gene expression analysis of VTA micropunches using RT-qPCR demonstrated that GHSR KOs had significantly decreased gene expression of the 2-AG synthesizing enzyme, DAGL- α (i.e. *Dagla*), relative to WTs ($t_{(7.165)} = 3.092$, $p = 0.017$, $d = 1.613$). In contrast, the gene expression of MAGL (i.e. *Mgll*), the predominant enzyme that breaks down 2-AG, did not significantly differ between GHSR KOs and WTs ($t_{(16)} = 0.649$, $p = 0.526$, $d = 0.308$). Furthermore, compared to WTs, GHSR KOs showed a trend towards increased gene expression of the AEA synthesizing enzyme, NAPE-PLD (i.e. *Napepld*) ($t_{(16)} = -1.856$, $p = 0.082$, $d = 0.995$), but reduced gene expression of the AEA degradative enzyme FAAH (i.e. *Faah*) ($t_{(17)} = 3.309$, $p = 0.004$, $d = 1.494$).

Figure 2.2: Genetic deletion of functional GHSRs tends to increase VTA *Napepld* but suppresses both *Dagla* and *Faah* gene expression relative to WT rats. Relative gene expression of the 2-AG synthesizing enzyme, *Dagla*, 2-AG degradative enzyme, *Mgll*, AEA synthesizing enzyme, *Napepld*, and AEA degradative enzyme, *Faah*, in GHSR KOs relative to WT. * *p* < .05.

2.4 Discussion

The present findings indicate that genetic disruption of functional GHSRs impacts the VTA endocannabinoid system and support the idea of an interaction between these two systems within this region. Specifically, these data show that rats with truncated GHSRs have reduced VTA 2-AG concentrations compared to WT animals. In support of this, gene expression analysis revealed that *Dagla*, the gene encoding the enzyme required for 2-AG production (i.e. DAGL- α), was downregulated relative to WT rats. In contrast, AEA levels did not differ between genotypes despite GHSR KOs demonstrating a trend for increased expression of *Napepld* and decreased expression of *Faah*, the genes encoding the enzymes responsible for AEA synthesis and degradation, respectively.

The decreased expression of *Dagla* and reduced concentration of 2-AG found in GHSR KOs relative to WT animals is consistent with our belief that GHSR activation leads to increased synthesis and release of 2-AG (Supplementary figure 2). GHSRs are expressed postsynaptically on dopaminergic (40–60%) and non-dopaminergic neurons (~30%) within the VTA (Abizaid et al., 2006b; Zigman et al., 2006). Activation of these receptors, induces PLC signaling cascades, augments intracellular calcium levels, and promotes DAG production (Yin et al., 2014). Interestingly, these effects are known to promote 2-AG biosynthesis and release from cells that express DAGL- α (Wang and Lupica, 2014). Appropriately, *Dagla* is expressed in a vast majority of neurons (dopaminergic and non-dopaminergic) within the VTA (Merrill et al., 2015). Consistent with this, immunostaining experiments locate DAGL- α on dendrites of both dopaminergic and non-dopaminergic neurons within the VTA (Mátyás et al., 2008). Although no one has specifically assessed what proportion of cells within the VTA co-express *Ghsr* and *Dagla* or their corresponding proteins (GHSRs and DAGL- α), given their respective prevalence within this region it is likely that they are co-expressed to some extent (Zigman et al., 2006; Mátyás et al., 2008; Mani et al., 2014; Merrill et al., 2015). Since GHSRs demonstrate high constitutive activity (i.e. they signal at 50% capacity even in the absence of ghrelin) in WT animals, our data demonstrating lower VTA *Dagla* expression and 2-AG levels in the GHSR KOs, where GHSR-

induced 2-AG promoting conditions are absent, are reasonable and consistent with a putative interaction between ghrelin and endocannabinoid systems within the VTA. In agreement with this, peripheral administration of ghrelin or raising ghrelin levels naturally through fasting enhances 2-AG levels within other feeding related brain regions that highly express GHSRs (e.g. HYP) (Kirkham et al., 2002; Zigman et al., 2006; Kola et al., 2008).

VTA AEA levels did not differ between genotypes despite gene expression data demonstrating a trend for higher expression of the AEA synthesizing enzyme *Napepld* and lower expression of the AEA degradative enzyme *Faah* in GHSR KO relative to WT. Several factors may contribute to the apparent disparity between the gene expression and AEA quantification data. First, *Faah* only encodes one of five enzymes able to degrade AEA (Glaser and Kaczocha, 2010; Luchicchi and Pistis, 2012). Therefore, the downregulation of *Faah* as well as the slight upregulation of *Napepld* in GHSR KO relative to WT may be compensated by an upregulation in other AEA degradative enzymes. Second, *Napepld* is sparsely expressed within the VTA (Merrill et al., 2015). Single-cell RT-qPCR techniques detect *Napepld* (at high cycle thresholds) in less than 40% of dopaminergic neurons and approximately 15% of GABA neurons selected within the VTA (Merrill et al., 2015). Given the aforementioned distribution and prevalence of GHSRs on dopaminergic and non-dopaminergic neurons within the VTA, it may be that the co-expression of GHSR in cells that express *Napepld* is insufficient for GHSR activation to substantially drive increases in AEA levels in WT (Abizaid et al., 2006b; Zigman et al., 2006; Merrill et al., 2015). In this case, AEA levels would not be expected to differ between GHSR KO and WT animals. Lastly, it is important to note that gene expression differences do not always translate into changes in protein, as posttranscriptional, translational, and degradation regulatory processes obfuscate this comparison (Vogel and Marcotte, 2013). It is estimated that only 40% of the variation in protein concentrations can reliably be predicted by mRNA expression data (Vogel and Marcotte, 2013). Accordingly, VTA NAPE-PLD and FAAH protein levels may remain relatively unchanged in GHSR KO relative to WT resulting in similar AEA concentrations between genotypes despite the gene expression differences detected.

It has become increasingly clear that 2-AG, which is estimated to be 100 to 1000 times more prevalent than AEA, is the predominant endocannabinoid regulating CB-1R-mediated synaptic plasticity in the brain (Bisogno et al., 1999; Alger and Kim, 2011; Luchicchi and Pistis, 2012). Appropriately, 2-AG is a full agonist while AEA is considered only a partial agonist at CB-1Rs (Howlett et al., 2002). Accordingly, genetic ablation of DAGL- α reduces 2-AG levels dramatically and abolishes CB-1R-mediated synaptic suppression of neurotransmitter release in many brain regions (Gao et al., 2010; Tanimura et al., 2010). This phenomenon also seems to be true within the VTA as studies similarly suggest that 2-AG is more prevalent than AEA (> 100) and the main endocannabinoid involved in regulating the activity of dopaminergic neurons that drive reward and motivated behaviours (Melis et al., 2004b; Pillolla et al., 2007; Oleson et al., 2012). Consistent with this, augmenting VTA 2-AG levels increases feeding motivation, cue evoked dopamine release, and reward seeking behaviours; however, augmenting AEA concentrations do not (Oleson et al., 2012). These data support our findings demonstrating that 2-AG concentrations were almost 1000-fold those of AEA within the VTA and that VTA 2-AG but not AEA levels were suppressed by the genetic loss of functional GHSRs.

In summary, our results demonstrate that genetic disruption of GHSR signaling induces significant changes to endocannabinoid system elements within the VTA. Most notably, loss of GHSR signaling significantly impairs the production of 2-AG within the VTA, which is an important effect given that 2-AG contributes to the modulation of VTA dopaminergic neurons that drive reward and motivated feeding behaviours. Although our gene expression and protein quantification data provide insufficient evidence to deduce the mechanism by which ghrelin and endocannabinoid systems interact within the VTA and the implications of this interaction (topics addressed in Chapters 3 and 4), the fact that disruption of the ghrelin system alters the endocannabinoid system within the VTA, strongly suggest that these systems interact within this region.

Chapter 3:

Assessing whether intra-VTA endocannabinoid signaling is required for ghrelin-driven food intake and motivated feeding behaviours

3.1 Rationale and overall approach

Engagement of ghrelin or endocannabinoid signaling promotes adiposity and feeding (Hao et al., 2000; Tschop et al., 2000; Jamshidi and Taylor, 2001; Wren et al., 2001b, 2001a; Kirkham et al., 2002; Williams and Kirkham, 2002). Appropriately, GHSRs and CB-1Rs are jointly expressed within peripheral organs associated with metabolism (i.e. adipose tissue and liver) and brain regions that regulate homeostatic (i.e. HYP) and non-homeostatic (i.e. VTA) feeding (Herkenham et al., 1991; Guan et al., 1997; Tsou et al., 1998; Mackie, 2005; Zigman et al., 2006; Mani et al., 2014). The functional overlap between ghrelin and endocannabinoid systems led to the discovery that these systems jointly modulate metabolism and feeding behaviours in various regions where their receptors are mutually expressed (Tucci et al., 2004; Kola et al., 2005, 2008; Lim et al., 2013). Consistent with this, disruption of GHSR signaling blocks the adipogenic effect of endocannabinoids within adipose tissue and the liver and the induction of orexigenic signaling cascades and feeding within the HYP (Lim et al., 2013). Reciprocally, disruption of CB-1R signaling likewise attenuates the adipogenic effect of ghrelin within both adipose tissue and the liver and its capacity to engage orexigenic circuits and promote feeding within the HYP (Tucci et al., 2004; Kola et al., 2005, 2008, 2013).

Despite the fact that GHSRs and CB-1Rs are jointly expressed within the VTA and that ghrelin and endocannabinoids similarly encourage non-homeostatic feeding within this region, the existence and importance of an interaction between ghrelin and endocannabinoid systems remains unclear within the VTA (Herkenham et al., 1991; Guan et al., 1997; Abizaid et al.,

2006b; Jarrett et al., 2007; Mátyás et al., 2008; Sinnayah et al., 2008; Oleson and Cheer, 2012; Mani et al., 2014; Edwards and Abizaid, 2016). Within this region, independent agonism of either GHSRs or CB-1Rs stimulates resident dopaminergic neurons, promotes dopamine release within the NA, and enhances motivated feeding behaviours (Tanda et al., 1997; Abizaid et al., 2006b; Solinas et al., 2006; Jerlhag et al., 2007; Sinnayah et al., 2008; King et al., 2011; Oleson et al., 2012; Oleson and Cheer, 2012; Cone et al., 2014; van der Plasse et al., 2015; Wang et al., 2015b). Given the overlapping functional roles of ghrelin and endocannabinoid systems in engaging these orexigenic neurocircuits and promoting motivated feeding behaviours within the VTA, we predicted, like demonstrated in the HYP, that these systems interact within the VTA and that a functional endocannabinoid system is necessary for driving ghrelin-induced non-homeostatic feeding within this region (Kola et al., 2008; Lim et al., 2013; Edwards and Abizaid, 2016).

In line with this prediction, we have previously demonstrated that manipulation of GHSR signaling modulates the endocannabinoid system within the VTA (Chapter 2) and that the orexigenic capacity of ghrelin within the VTA can be abolished by global CB-1R antagonism (i.e. peripheral administration of a CB-1R antagonist) (Edwards, 2014). These data support a putative interaction between ghrelin and endocannabinoid systems within the VTA and suggest that intra-VTA ghrelin requires a functional endocannabinoid system to promote feeding within this region. However, one cannot conclude that the feeding effects observed were due to an interaction of these systems specifically within the VTA as the CB-1R antagonist was administered peripherally in the latter experiment. Therefore, despite the commonalities between ghrelin and endocannabinoid systems within the VTA and our circumstantial evidence suggesting that these systems interact within the VTA to modulate feeding, it has yet to be conclusively determined if these systems interact specifically within the VTA to modulate non-homeostatic feeding behaviours.

To address this, we first explored if blocking CB-1Rs specifically within the VTA (intra-VTA microinfusion of rimonabant) could attenuate the rise in food intake and locomotor

activity that accompanies intra-VTA infusion of ghrelin in satiated rats (Abizaid et al., 2006b; Jerlhag et al., 2007; Skibicka et al., 2013). Furthermore, as ghrelin and endocannabinoids are predominantly thought to increase food intake within the VTA by enhancing the motivation to feed (Solinas and Goldberg, 2005; King et al., 2011; Weinberg et al., 2011; Oleson et al., 2012; Skibicka et al., 2013), we also assessed if CB-1R signaling was necessary for intra-VTA ghrelin to enhance efforts to obtain food rewards using a progressive ratio paradigm.

3.2 General methods

3.2.1 Animals

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. Experiments were run at Carleton University and the University of Ottawa under temperature (22°C) and humidity controlled (45–55%) conditions. Long Evans rats (260–300 g; 7–9 weeks) were purchased from Charles River for both food intake and operant feeding studies. Rats were housed individually and were given *ad libitum* access to standard lab chow (Harlan Diets; 3.3 kcal/g; 4% fat; 48% carbohydrates; 14.3% protein) and water unless otherwise stated (i.e. operant training paradigm). Rats were given a one-week post-delivery habituation period before presurgery baseline food consumption and body weight measurements began. The presurgery body weight and food intake measurements were taken daily at the beginning of the light cycle and persisted for a minimum of one week. Rats were subjected to stereotaxic surgery to implant a guide cannula above the VTA after this one-week baseline period (i.e. feeding study) or after successful operant training (i.e. feeding motivation study). Following stereotaxic surgery, rats were given at least one week to recover. For both studies, fully recovered rats were sorted into one of the following four pharmacological treatment groups: rimonabant (0.5 µg) /ghrelin (1 µg), rimonabant (0.5 µg)/saline, vehicle/ghrelin (1 µg), or vehicle/saline. Subjects were sorted into treatment groups such that body weight and food

intake averages did not differ between treatment groups (i.e. matched subjects design). Rats were handled daily throughout these experiments to minimize stress during experimental manipulations.

3.2.2 Intra-VTA cannula surgical procedures

Anesthesia was induced and maintained by exposing rats to an isoflurane:oxygen gas mixture of 5:2 and 2.5:2, respectively. Metacam (5 mg/ml) was intraperitoneally administered (0.5 mg/kg) prior to the initiation of surgery to ensure adequate analgesia during and postsurgery. Scalps were shaved and cleaned with germistat, alcohol, and providine to maintain an aseptic canvass around the incision site and tear gel was applied to prevent dehydration of the eyes. Rats were secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) and adequate anesthetic depth was confirmed before a midline incision was made. Hemostats were used to retract the skin and the anterior to posterior (AP –5.6 mm) and medial to lateral (ML +2.0 mm) coordinates of the VTA relative to bregma were measured and delineated on the skull. A surgical drill was used to bore holes in the skull for implantation of the unilateral guide cannula and associated anchoring screws. Anchoring screws were affixed and the guide cannula, angled towards the midline (10°) to avoid the aqueduct, was inserted to a depth just above the VTA (DV –7.8 mm). Dental cement was molded around the cannula and anchoring screws to stabilize and prevent movement of the guide cannula throughout behavioural experiments. Once dry, the incision was sutured and a dummy cannula was inserted into the guide cannula to mitigate the chances of clogging. Rats were placed in freshly clean cages and monitored closely for at least two hours after surgery for signs of surgical complications. Rats were treated post-operatively with subcutaneous Metacam (0.5 mg/kg) for a minimum of two days after surgery and were monitored twice daily for a least a week to ensure optimal recovery. Only animals deemed fully recovered (i.e. asymptomatic, stable food intake, gaining weight) were assigned to behavioural experiments.

3.2.3 Drugs

Rimonabant hydrochloride (Sigma Aldrich, St.Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.9% saline to a final concentration of 1 µg/µl (final concentration of 5% DMSO). The rimonabant solution was heated and sonicated to ensure a homogenous distribution of rimonabant in solution before its microinfusion into the VTA. Acylated rat ghrelin (Tocris Bioscience, Bristol, United Kingdom) was diluted in 0.9% saline to a final concentration of 2 µg/µl for microinfusion injections. A 5% (v/v) solution of DMSO in 0.9% saline was used as the vehicle solution for the rimonabant antagonist treatment while 0.9% saline was used as the control treatment for ghrelin administration.

3.2.4 Experiment 1: Food intake and locomotor activity assessment

Rats were assigned to treatment groups such that postsurgery body weights and daily food intake were matched across treatments. On test day, freely behaving VTA-cannulated rats were momentarily restrained to facilitate insertion of the infusion cannula into the guide cannula. Rats were placed back in their home cage while a GenieTouch™ dual syringe microinfusion pump (Kent Scientific, Torrington, CT) infused 0.5 µl of their first treatment (i.e. vehicle or rimonabant) over a 2-minute time period. Infusion cannulas were left in place for 30 seconds after microinfusion to ensure drug delivery before removal. Rats were given a 30-minute home cage break to facilitate diffusion of their first microinfusion. After this break, rats were given their second microinfusion (i.e. saline or ghrelin) using identical methodologies. Accordingly, each animal received one of the following four treatments: rimonabant (0.5 µg)/ghrelin (1 µg), rimonabant (0.5 µg)/saline, vehicle/ghrelin (1 µg), or vehicle/saline. Each treatment consisted of two separate 0.5 µl microinjections (infused over 2 minutes), spaced 30 minutes apart (refer to Supplementary figure 3). Immediately following the second microinfusion, rats were transferred to a clean home cage equipped with a known amount of standard chow pellets and water. A Home Cage Activity System (Omnitech Electronics Incorporated, Dartmouth, NS) was used to monitor overall locomotor activity (i.e. beam breaks) throughout the feeding study to examine whether differences in food intake could be attributed to treatment-induced increases or decreases in locomotor activity. The amount of

food consumed and locomotor activity were measured 30, 60, 120, 240, and 360 minutes after the second microinfusion. All rats received mock infusions for one week before test day to minimize procedural-induced stress.

3.2.5 Experiment 2: Operant conditioning procedure and feeding motivation assessment

Operant conditioning training and experiments were conducted in standard operant conditioning chambers (height x width x depth = 13 x 10 x 12 inches; Colbourn Instruments, Allentown, PA) equipped with: two levers (i.e. active and inactive), a pellet dispensing hopper, a house light, and a grid floor. Active and inactive lever presses as well as overall locomotor activity were recorded by Graphic State (version 3) software (Colbourn Instruments). For operant conditioning training and experiments, rats received a standard chow-like 45 mg Dustless Precision Pellet (Bio Serv, Flemington, New Jersey; 3.6 kcal/g; 5.6% fat; 59.1% carbohydrates; 18.7% protein) upon satisfying the scheduled number of active lever presses required to obtain a reward. A timeline of the operant conditioning training and experimental design is depicted in Appendix A (Supplementary figure 4).

Male Long Evans rats were allowed to habituate before food intake and body weight baseline measurements were recorded. They were then food-restricted to 90% of their baseline body weight to facilitate operant conditioning training. Rats were first trained to bar press on a fixed-ratio 1 (FR-1) schedule where each depression of the active lever resulted in the delivery of a single food pellet. These training sessions were 30 minutes in duration and were conducted during the first half of the light cycle. Rats received their respective training session at the same time for the duration of operant conditioning training. Once their responding stabilized (i.e. less than 15% variation in active lever presses between three consecutive training sessions), they were moved up to a FR-3 schedule, where three active lever presses were required to obtain the food rewards. Similarly, once rats were stably responding on a FR-3 schedule, they were graduated to a FR-5 schedule, where five active lever presses were required to obtain food rewards. On average, rats required 7–10 days to graduate from a FR-1 to a FR-3 and 3–5 days to

reach the FR-5 schedule. After reaching FR-5 responding stability guidelines, rats were given *ad libitum* food and water for 3 days prior to their stereotaxic surgery (i.e. implantation of guide cannula above the VTA). Rats were given at minimum 7 days to recover from surgery; however, they were not readmitted to operant training procedures until sufficiently recovered. Once recovered, they were again food restricted to 90% of postsurgery recovery weight (i.e. day 7) and given four FR-5 training sessions to ensure that the surgery did not disrupt their capacity to bar press. Rats with no surgical complications and stable FR-5 responding rates were subjected to mock microinfusions and given one practice PR training session to acclimate them to both the microinfusion procedure and to a schedule where the number of active lever presses required to obtain food rewards progressively increases. The PR schedule used (i.e. 1, 2, 4, 6, 9, 11, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, etc.) was developed by Richardson and Roberts (2006) to assess the efficacy of a reinforcer (e.g. drugs) at promoting motivated behaviours. The schedule was derived from the following equation: response ratio (rounded to nearest integer) = $[5e^{(\text{number of rewards obtained} \times 0.2)}] - 5$ (Richardson and Roberts, 1996). Practice and final test PR sessions ended when rats failed to obtain their next reward within 30 minutes of their previous reward. The amount of food rewards obtained before a rat gave up and timed out was defined as their breakpoint and was taken as an index of how motivated a rat was to work for food rewards. Rats were sorted into four groups after their practice PR session so that the average number of active lever presses across the last three FR-5 training sessions and within the practice PR session, did not differ between treatment groups. After the PR practice session, rats were given *ad libitum* food and water for three days prior to their final PR test. On test day, each group was randomly assigned to one of the following treatments: rimonabant (0.5 µg)/ghrelin (1 µg), rimonabant (0.5 µg)/saline, vehicle/ghrelin (1 µg), or vehicle/saline. These combinatorial treatments were given via two separate 0.5 µl microinjections (infused over 2 minutes), spaced 30 minutes apart, as described previously (section 3.2.4). Immediately following the second microinfusion, rats were placed in the operant chamber for their final PR test session to assess treatment-induced changes in active lever pressing (i.e. motivation to feed).

3.2.6 Histological analyses and cannula placement verification

Upon completion of behavioural experiments, rats were overdosed with urethane (1 g/kg) and euthanized via transcardial perfusion. Isotonic saline was circulated to clear the blood before circulation of a 4% paraformaldehyde (PFA) fixative solution. Following fixation, rats were quickly decapitated and brains were extracted. Brains were incubated in 4% PFA for an additional 48 hours and then transferred to a 30% sucrose solution: 0.1 M phosphate buffer cryoprotectant solution (weight/volume). Brains were left in cyroprotectant at 4°C until adequately dehydrated (2–3 days). Following dehydration, brains were frozen and sliced coronally on a CM1900 cryostat (Leica Biosystems Inc., Concord, ON) into 50 µM sections. The position of the tip of the cannula in each rat brain was diligently noted and classified as either falling within or out of the delineations of the VTA (Figure 3.1A). Data from rats with well-positioned cannulae (represented as green dots) were included in all analyses; whereas, data from rats with missed cannulae (represented as orange dots) were excluded from all analyses. Data from rats with well-positioned cannulae but whom displayed sickness or erratic (extreme outliers) behaviours (represented as grey dots) were also excluded from analyses.

3.2.7 Data Analyses

Food intake and locomotor activity (dependent variable) data was analyzed by two-way analysis of variance (ANOVA) at each of the specified time points (30, 60, 120, 240, and 360 minutes) with the antagonist treatment (i.e. rimonabant or vehicle) as one independent factor and the agonist treatment (i.e. ghrelin or saline) as the second independent factor. Significant interaction effects were followed up with an analysis of the simple main effects and associated post-hoc pairwise comparisons to evaluate which treatment groups significantly differed from one another. Likewise, PR breakpoints and the total number of active lever presses between groups were also analyzed with two-way ANOVAs. Although, interactions between the antagonist and agonist treatment did not reach statistical significance (largely due to inadequate power associated with small number of rats within each group), since *a priori* predictions concerning this interaction were made and that the partial η^2 squared for these

interactions indicated that a moderate amount of the variances were accounted for, follow up tests were conducted of the simple main effects comprising these interactions. Main effects were also examined for operant experiments. Interactions, main effects, and post-hoc tests were conducted with $p < .05$ being considered statistically significant. Animals with misplaced cannulas or that displayed erratic (extreme outliers) or sickness behaviours (extreme outliers) following microinfusion were excluded from analysis. Furthermore, data were excluded from analyses if equipment malfunctioned (i.e. home cage activity boxes or conditioning boxes). All statistics were completed using IBM SPSS 21 statistical software. Results were represented as the mean $\pm$ S.E.M.

3.3 Results

3.3.1 Intra-VTA rimonabant pretreatment blocks ghrelin-induced acute feeding within the VTA

The capacity of ghrelin to increase food intake when infused into the VTA depended on whether rats received intra-VTA vehicle or rimonabant pretreatment (interaction between pretreatment and treatment) (Figure 3.1B). This statistically significant interaction effect manifested two hours ($F_{(1, 37)} = 5.197$, $p = .028$, partial $\eta^2 = .123$) after the second microinfusion; however, maximal effects occurred four ($F_{(1, 37)} = 7.615$, $p = .009$ partial $\eta^2 = .171$) and six ($F_{(1, 37)} = 8.000$, $p = .008$, partial $\eta^2 = .178$) hours after. Follow-up post-hoc tests of the simple main effects comprising these interactions demonstrated that while ghrelin potentially encouraged food intake on its own, rimonabant pretreatment mitigated its capacity to do so. Accordingly, at the two-hour time point, rats that received ghrelin alone (vehicle/ghrelin, $n = 13$) tended to consume more than saline controls (vehicle/saline, $n = 11$) ($p = .100$) and those that were infused with rimonabant prior to ghrelin (rimonabant/ghrelin, $n = 7$) ($p = .089$). These effects became more apparent at the four- and six-hour time points as vehicle/ghrelin treated animals ate significantly more than rimonabant/ghrelin (4 hr, $p = .034$; 6 hr, $p = .013$) treated and vehicle/saline (4 hr, $p = .007$; 6 hr, $p = .012$) controls. Importantly, food intake of

rimonabant/saline ($n = 10$) and rimonabant/ghrelin treated animals did not differ from vehicle/saline controls at any of the time points tested ($p > .05$). Twenty-four-hour food consumption (data not shown) also did not differ between treatment groups.

In line with the treatment-induced feeding effects, the capacity of ghrelin to stimulate locomotor activity (Figure 3.1C) depended on whether rats received intra-VTA vehicle or rimonabant pretreatment (interaction between pretreatment and treatment). This interaction emerged at the two-hour time point ($F_{(1, 37)} = 3.931$, $p = .055$, partial $\eta^2 = .101$) but did not reach statistical significance until the four-hour ($F_{(1, 37)} = 4.849$, $p = .034$, partial $\eta^2 = .122$) and six-hour ($F_{(1, 37)} = 4.393$, $p = .043$, partial $\eta^2 = .112$) time points. Accordingly, while rats that received ghrelin alone (vehicle/ghrelin, $n = 12$) tended to move more than saline controls (vehicle/saline, $n = 10$) at the four- ($p = .066$), and six-hour ($p = .089$) time points, ghrelin treated rats that were pretreated with rimonabant (rimonabant/ghrelin, $n = 7$) did not significantly differ from other treatments with respect to locomotor activity ($p > .05$).

Figure 3.1: Intra-VTA CB-1R antagonism prevents intra-VTA ghrelin-induced food intake in non-fasted male Long Evans rats. **(A)** Summary of cannulae placements depicting well-positioned (green dots), missed (orange dots), and well-positioned but removed (grey dots) cannulae placements. Cumulative home cage **(B)** food intake and **(C)** locomotor activity following corresponding intra-VTA pharmacological treatments. Rats were infused (0.5 μ l each) with either saline or rimonabant (0.5 μ g) 30 minutes prior to saline or ghrelin (1 μ g). * $p < .05$.

3.3.2 Intra-VTA CB-1R antagonism blunts the capacity of ghrelin to increase motivated feeding behaviours within the VTA

Intra-VTA ghrelin administration significantly increased breakpoints (Figure 3.2B, main effect of ghrelin: $F_{(1, 23)} = 6.389$, $p = .019$, partial $\eta^2 = .217$) and total active lever presses (Figure 3.2C, main effect of ghrelin: $F_{(1, 23)} = 6.942$, $p = .015$, partial $\eta^2 = .232$) until breakpoint relative to saline treated animals in the PR task suggesting potentiated feeding motivation in ghrelin-treated rats. In contrast, intra-VTA rimonabant administration did not significantly alter breakpoints (no main effect of rimonabant: $F_{(1, 23)} = 0.239$, $p = .630$, partial $\eta^2 = .010$) or total active lever presses until breakpoint (no main effect of rimonabant: $F_{(1, 23)} = 1.276$, $p = .270$, partial $\eta^2 = .053$). The interaction between rimonabant and ghrelin treatments did not reach statistical significance for either breakpoint ($F_{(1, 23)} = 0.997$, $p = .328$, partial $\eta^2 = .042$) or active lever presses prior to breakpoint ($F_{(1, 23)} = 1.408$, $p = .248$, partial $\eta^2 = .058$), likely due to inadequate power associated with the relatively small number of rats within groups; however, since *a priori* predictions concerning this interaction were made and since the partial η^2 for the interaction indicates that a moderate amount of the variance was accounted for, follow up tests were conducted of the simple main effects comprising these interactions. Importantly, although vehicle/ghrelin ($n = 6$) and rimonabant/ghrelin ($n = 7$) groups did not significantly differ with respect to their breakpoint ($p = .313$) or total active lever presses prior to breakpoint ($p = .122$), follow up tests indicated that while intra-VTA ghrelin produced a significant increase in both breakpoint ($p = .023$) and total active lever presses prior to breakpoint ($p = .014$) when animals were pretreated with DMSO (relative to vehicle/saline ($n = 7$) controls), ghrelin did not significantly increase breakpoints ($p = .281$) or total active lever presses ($p = .307$) when animals were pretreated with rimonabant (relative to rimonabant/saline controls ($n = 7$)).

Intra-VTA administration of ghrelin tended to increase locomotor activity (Figure 3.2D) of rats during the PR task; however, this effect did not reach statistical significance (trend for main effect of ghrelin $F_{(1, 23)} = 3.898$, $p = .061$, partial $\eta^2 = .145$). In contrast, locomotor activity in the PR task was unaffected by intra-VTA administration of rimonabant (no main effect of

rimonabant: $F_{(1, 23)} = 0.116$, $p = .736$, partial $\eta^2 = .005$). Although, no rimonabant and ghrelin treatment interaction was observed with respect to locomotor activity (no interaction $F_{(1, 23)} = 0.574$, $p = .456$, partial $\eta^2 = .024$), since *a priori* predictions concerning this interaction were made and that a moderate amount of the variance was once again accounted for by the interaction, follow up tests were conducted of the simple main effects comprising this interaction. Accordingly, while vehicle/ghrelin and rimonabant/ghrelin groups did not significantly differ with respect to their locomotor activity ($p = .775$), ghrelin-treated rats tended to increase their locomotor activity compare to corresponding controls when rats were pretreated with DMSO ($p = .071$) but not rimonabant ($p = .389$).

Figure 3.2: Intra-VTA CB-1R antagonism blunts intra-VTA ghrelin-induced increases in feeding motivation as assessed in progressive ratio experiments using non-fasted male Long Evans rats. **(A)** Summary cannulae placements depicting well-positioned (green dots), missed (orange dots), and well-positioned but removed (grey dots) cannulae placements. Progressive ratio test **(B)** breakpoints, **(C)** active lever presses, and **(D)** locomotor activity following corresponding intra-VTA pharmacological treatments. Rats were infused (0.5 μ l per injection) with either saline or rimonabant (0.5 μ g) 30 minutes prior to saline or ghrelin (1 μ g). * $p < .05$.

3.4 Discussion

The present findings reveal a previously unrecognized role for CB-1R signaling in mediating ghrelin-induced motivated feeding behaviours within the VTA. These data reaffirm the potent orexigenic and motivational capacity of intra-VTA ghrelin. Importantly, we show that these intra-VTA ghrelin effects rely on CB-1R signaling as the robust feeding effects induced by intra-VTA ghrelin were attenuated by pharmacological blockade of VTA CB-1Rs. Moreover, we demonstrated that the treatment-induced feeding effects may, in part, be regulated by alterations in locomotor activity. Given that the VTA plays an important role in regulating motivated behaviours, we subsequently examined the possibility that intra-VTA CB-1R antagonism may block ghrelin-induced feeding by attenuating the increased feeding motivation that traditionally accompanies intra-VTA ghrelin administration (King et al., 2011; Skibicka et al., 2013). In support of this, progressive ratio experiments for food rewards showed enhanced break points, active lever presses, and a trend for increased locomotor activity when rats were administered intra-VTA ghrelin alone but not when pretreated with a CB-1R antagonist into the VTA prior to ghrelin.

The significant increases in food intake observed in this study when rats were administered intra-VTA ghrelin alone (i.e. vehicle/ghrelin) compared to saline controls (i.e. vehicle/saline) are consistent with previous data demonstrating voracious feeding in rodents following intra-VTA ghrelin administration (Naleid et al., 2005; Abizaid et al., 2006b; King et al., 2011; Skibicka et al., 2013; Wei et al., 2015; Kalafateli et al., 2018). Although the mechanisms underlying the ghrelin-induced feeding effect were not assessed here (see Chapter 4), previous reports suggest that the orexigenic action of ghrelin is predominantly driven by its capacity to stimulate VTA dopaminergic neurons and heighten subsequent dopamine release within mesocorticolimbic targets such as the NA (Abizaid et al., 2006b; Chuang et al., 2011; Weinberg et al., 2011; Skibicka et al., 2013). Ghrelin stimulates these VTA dopamine neurons directly by binding to excitatory G-protein-coupled GHSR receptors located on their surface and indirectly by increasing excitatory and reducing inhibitory tone onto them (Abizaid et al., 2006b). The fact

that intra-VTA ghrelin, in this study, stimulated food intake in free feeding rats at the beginning of the light cycle (i.e. when rats were presumably satiated) is congruent with previous reports showing that ghrelin excites these VTA dopamine neurons to drive feeding in the absence of hunger (Naleid et al., 2005; Abizaid et al., 2006b; King et al., 2011; Kalafateli et al., 2018).

The time course of the intra-VTA ghrelin-induced feeding observed herein differed slightly from previous reports looking at acute food intake (Naleid et al., 2005; Abizaid et al., 2006b). In this study, the orexigenic effect of ghrelin in vehicle/ghrelin treated rats began to emerge two hours after its infusion into the VTA relative to vehicle/saline controls; however, it did not significantly do so until four hours after its infusion. In contrast, others have detected significant and clear increases in food consumption as early as one hour (Naleid et al., 2005) or two hours (Abizaid et al., 2006b) after intra-VTA ghrelin administration. The discrepancy between our intra-VTA ghrelin-induced time course and that of Naleid and colleagues (2005) is likely procedural in nature. Naleid and colleagues (2005) bilaterally administered twice as much ghrelin than unilaterally administered in the present study. Accordingly, the quicker manifestation of ghrelin-induced feeding effects observed by Naleid and colleagues likely reflects a more robust ghrelin-induced activation of VTA dopaminergic neurons (Naleid et al., 2005). The reasons underlying the slightly delayed orexigenic action of ghrelin in our study compared to that observed by Abizaid et al. (2006b) are puzzling given that Abizaid and colleagues used a weaker dose of ghrelin than that used herein. This delay may stem from the fact that rats herein received two separate microinfusions, whereas rats were given a sole microinfusion in the feeding study conducted by Abizaid and colleagues. The stress associated with two rather than a single infusion may have played a role in the observed delay. Aside from these methodological difference other factors remained largely consistent (e.g. coordinates, unilateral infusion, ghrelin volume); however, slight differences in cannula placement within the VTA and/or rat strain differences may have also contributed. Consistent with this, others have shown that bregma, which is often used as a reference point for cannula placements, significantly varies between Long Evans (used in present experiments) and Sprague Dawley rats (used by Abizaid and colleagues) and that different rat strains commonly display subtle

behavioural differences (Kline and Reid, 1984; Clemens et al., 2014; Ku et al., 2016). Nonetheless, despite mild differences in the onset of food intake following ghrelin administration our data are consistent with previous studies demonstrating that ghrelin potentially promotes non-homeostatic food intake when infused into the VTA of rodents.

In addition to the heightened food consumption seen in vehicle/ghrelin relative to vehicle/saline controls, we observed a concomitant trend for increased locomotor activity in ghrelin-treated animals. These data are compatible with previous work demonstrating that intra-VTA ghrelin significantly stimulates locomotor activity both when food is freely available (Egecioglu et al., 2010) and when it is not (Jerlhag et al., 2007; Kalafateli et al., 2018). The stimulation of locomotor activity seen following intra-VTA ghrelin infusion has largely been attributed to enhanced NA dopamine release initiated by GHSR-mediated activation of dopaminergic VTA neurons (Jerlhag et al., 2007, 2009; Egecioglu et al., 2010; Jerlhag and Engel, 2011). Congruent with this, hyperlocomotor activity commonly accompanies conditions known to increase dopamine release within the NA (e.g. administration of drugs of abuse such as amphetamine and cocaine) (Willuhn et al., 2010).

Although the ability of ghrelin to increase locomotor activity surely contributes to intra-VTA ghrelin-induced food intake, we and others have shown ghrelin-induced increases in feeding without significant increases in locomotor activity indicating that other factors (e.g. enhanced motivation) likewise play an important role (Tang-Christensen et al., 2004; Edwards, 2014). Moreover, it is important to note that those that have reported strong ghrelin-induced increases in locomotor activity, bilaterally administered ghrelin at an equivalent or higher dose than unilaterally administered here (Jerlhag et al., 2007; Egecioglu et al., 2010). In addition, in these previous studies rodents were administered a single infusion, whereas rodents herein received two microinfusions. Therefore, the stronger induction of locomotor activity following intra-VTA ghrelin administration observed in other studies compared to those reported here may reflect a stronger or more robust activation of VTA dopaminergic neurons in the former or may simply reflect slight procedurally-induced alterations in locomotor activity. Nonetheless,

our data demonstrating a similar and concomitant increase in feeding and locomotor activity in response to intra-VTA ghrelin lend support to previous claims that increased locomotor activity may contribute to the orexigenic action of ghrelin within the VTA.

Rimonabant is renowned for its capacity to reduce appetite (Colombo et al., 1998; Simiand et al., 1998; Koch, 2017). We purposefully selected a subanorectic dose of rimonabant in our feeding and locomotor experiments to mitigate putative side effects (e.g. anxiety) (Haller et al., 2002) and to more delicately examine if simultaneous manipulation of ghrelin and endocannabinoid systems detectably impact feeding (Gomez et al., 2002; Tallarida, 2011). Unexpectedly, at this dose rimonabant appeared to potentiate feeding and locomotor activity compared to controls (i.e. rimonabant/saline versus vehicle/saline); however, these effects did not reach statistical significance. The reasons underlying these counterintuitive and weak increases in feeding and locomotor activity following rimonabant administration are unknown but have been observed previously (Edwards, 2014). In agreement with this, despite a general consensus that CB-1R antagonism decreases locomotor activity, others have also reported increases in locomotor activity when rimonabant is injected into the VTA alone (although at higher doses than used herein) (Kalafateli et al., 2018). Together these data suggest that at times CB-1R antagonism may excite VTA dopaminergic neurons (Kalafateli et al., 2018). Although CB-1R antagonism is for the most part thought to reduce the excitability of VTA dopaminergic neurons by preventing endocannabinoids from dampening GABAergic tone onto them; others have demonstrated that CB-1R antagonism, in some instances, is also capable of increasing the neuronal excitability of these VTA dopaminergic neurons by preventing endocannabinoid-mediated shut down of glutamatergic tone onto them (Szabo et al., 2002; Melis et al., 2004b, 2004a; Lupica and Riegel, 2005). While this may be the case here, caution should nevertheless be taken in interpreting these data, given the apparent but non-significant effect that rimonabant had on feeding and locomotor activity.

Our finding that intra-VTA rimonabant pretreatment completely abolished ghrelin-induced feeding and locomotor activity within the VTA to a level indistinguishable from

controls, highlights a previously unrecognized role of CB-1R signaling in mediating the orexigenic action of ghrelin within the VTA. Furthermore, these data support the notion that increased locomotor activity may contribute to the endocannabinoid-mediated orexigenic action of ghrelin within the VTA. These findings are consistent with the known ability of ghrelin to activate VTA dopaminergic neurons, enhance extracellular NA dopamine, and promote food intake and food-seeking behaviours when infused into VTA (Abizaid et al., 2006b). Moreover these data are also congruent with the capacity of CB-1R antagonists to oppose these effects (Sinnayah et al., 2008; Oleson and Cheer, 2012; Oleson et al., 2012). The fact that VTA CB-1R antagonism was shown herein to sufficiently block ghrelin-driven behaviours within the VTA is consistent with previous reports that demonstrate a loss of ghrelin-induced metabolic and feeding effects upon disruption of CB-1R signaling (Tucci et al., 2004; Kola et al., 2005, 2008, 2013; Alen et al., 2013). Most notably, the inability of ghrelin to engage orexigenic neurocircuits or promote food intake within the HYP when CB-1Rs are pharmacologically or genetically disrupted (Tucci et al., 2004; Kola et al., 2008).

Nevertheless, to our knowledge our findings are odds with, the only other study that has specifically examined the extent to which CB-1R antagonism impacts the orexigenic actions of ghrelin within the VTA (Kalafateli et al., 2018). In contrast to our findings, Kalafateli and colleagues (2008) found that intra-VTA rimonabant pretreatment was unable to attenuate ghrelin-induce increases in standard chow consumption four hours after intra-VTA microinfusions. Many factors may underlie or contribute to the disparate feeding findings. First, we conducted our experiments in rats, whereas Kalafateli et al. (2018) used mice; therefore, despite the fact that ghrelin and endocannabinoids are known to increase food intake, enhance the activity of VTA dopaminergic neurons, and augment NA dopamine release in both rats and mice, one cannot exclude the possibility that species differences may underlie the contradictory feeding results (Abizaid et al., 2006b; Solinas et al., 2006; Sinnayah et al., 2008; Oleson et al., 2012; Kalafateli et al., 2018). Related to this, since the magnitude of chow consumed over a four-hour period is much greater for rats than it is for mice, it follows that treatments that modulate food intake would be more apparent and detectable in the former. For example, in

our study, ghrelin-treated rats ate approximately two grams more than vehicle controls four hours post injection, whereas Kalafateli and colleagues (2018) found that ghrelin and vehicle treated mice differed by only 0.2 grams. While this does not diminish the significance of their results, it does limit their threshold for detecting a change in ghrelin-induced feeding with rimonabant pretreatment. Lastly, the disparate results between our study and Kalafateli et al. (2018) could have been driven by differences in the feeding setup and/or procedure. Notably, we used a much lower concentration of DMSO (i.e. 5%) in the pretreatment solutions (vehicle and rimonabant) compared to that used by Kalafateli and colleagues (i.e. 75%) to mitigate putative DMSO-induced behavioural effects. Although some argue that DMSO concentrations of up to 75% are acceptable for ICV injections (Blevins et al., 2002), others report behavioural and/or deleterious DMSO-induced effects at much lower concentrations (Castro et al., 1995; Hanslick et al., 2010; Yuan et al., 2014). In addition, Kalafateli and colleagues placed mice in a new cage one hour prior to infusions and presented chow in a plastic cup, whereas in our study rats were infused and placed back in their home cage with food presented in traditional feeding hoppers. Novelty-induced suppression of feeding behaviours in the former may have made it harder to detect treatment-induced differences in food consumption. In support of this, independent of combinatorial treatment received, the total amount of food consumed within the four-hour feeding test was quite low in Kalafateli et al. (2018) relative to other studies examining feeding in *ad libitum* fed mice after central microinfusions (i.e. ICV or intra-VTA) (Sinnayah et al., 2008; Jerlhag et al., 2011b; Schéle et al., 2016).

Despite the procedural and feeding data differences, our treatment-induced locomotor activity data demonstrating that intra-VTA CB-1R antagonism was sufficient to block ghrelin-induced increases in locomotor activity are in agreement with those reported by Kalafateli and colleagues (Kalafateli et al., 2018). This corroborates previous reports showing an attenuation of drug-induced (e.g. cocaine, ethanol), VTA dopaminergic neuron driven hyperlocomotor activity and extracellular NA dopamine release following pharmacological or genetic disruption of CB-1R signaling (Cheer et al., 2007; Li et al., 2008; Marinho et al., 2015). Kalafateli et al. (2018) argued that cannabinoid signaling, within the VTA, is important for ghrelin-induced

locomotor activity but not ghrelin-induced feeding. However, given our data and that these behaviours are encouraged by similar cellular processes (i.e. activation of VTA dopaminergic neurons and enhanced dopamine release within the NA), we alternatively suggest that their low feeding levels across groups may have prevented them from resolving an interaction between these systems with respect to feeding (Abizaid et al., 2006b; Jerlhag et al., 2007, 2009; Sinnayah et al., 2008; Oleson et al., 2012; Kalafateli et al., 2018). Nonetheless, the fact that Kalafateli and colleagues observed an attenuation of ghrelin-induced locomotor activity upon rimonabant pretreatment agrees with our data and our belief that ghrelin and endocannabinoid systems interact within the VTA to modulate feeding behaviours.

In addition to our findings demonstrating an abolished capacity of ghrelin to induce feeding and locomotor activity when intra-VTA CB-1Rs were antagonized, our PR studies revealed that the enhanced motivation to feed that accompanies intra-VTA ghrelin is similarly attenuated when CB-1Rs are blocked within this region. Accordingly, the heightened breakpoints, active lever presses, and locomotor activity of rats administered intra-VTA ghrelin alone but not those pretreated with rimonabant, reaffirms the strong capacity of ghrelin to enhance efforts (i.e. motivation) to obtain food rewards (Davis et al., 2007; Jerlhag, 2008; Perello et al., 2010; King et al., 2011; Skibicka et al., 2011, 2013) and identifies the endocannabinoid system as an important mediator of these effects within the VTA. These data are compatible with studies that demonstrate that intra-VTA ghrelin potently increases active lever presses and breakpoints for foods (King et al., 2011; Skibicka et al., 2011, 2013) and those showing reduced feeding motivation (e.g. decreased breakpoints, active lever presses, reward seeking) upon peripheral and intra-tegmental CB-1R antagonism (Perio et al., 2001; Solinas and Goldberg, 2005; Oleson et al., 2012). Importantly, intra-VTA injection of rimonabant alone (at a low subanorectic dose) did not impact feeding motivation or locomotor activity relative to controls in the progressive ratio task. This indicates that in the absence of ghrelin, blocking CB-1Rs and suppressing tonic endocannabinoid signaling does not significantly impact these behaviours. Together, these data highlight that CB-1R signaling is important for mediating ghrelin-induced motivated feeding behaviours within the VTA.

In summary, our results demonstrate that pharmacological disruption of CB-1R signaling within the VTA sufficiently attenuates the capacity of intra-VTA ghrelin to increase locomotor activity, food intake, and the motivation to feed. Together, these studies have identified a previously unrecognized role for CB-1R signaling in mediating ghrelin-induced motivated feeding behaviours within the VTA.

Chapter 4:

Probing the mechanism of interaction between ghrelin and endocannabinoid systems within the VTA via electrophysiology

4.1 Rationale and overall approach

Ghrelin and endocannabinoids are known to promote the activity of dopaminergic neurons within the VTA to increase motivational processes and encourage behaviours important for obtaining and consuming food (Tanda et al., 1997; Solinas and Goldberg, 2005; Abizaid et al., 2006b; Solinas et al., 2006; Perello et al., 2010; King et al., 2011; Oleson et al., 2012; Edwards, 2014). The data presented and discussed in the preceding chapters provide compelling evidence that ghrelin and endocannabinoid systems interact within the VTA and that this interaction is important for regulating non-homeostatic feeding behaviours. Specifically, we have demonstrated that ghrelin loses its capacity to elicit motivated feeding behaviours within the VTA when VTA CB-1Rs are antagonized suggesting that CB-1R signaling mediates ghrelin-induced increases in motivated feeding behaviours within this region (Chapter 3). Given this, we propose that CB-1R signaling may be required for ghrelin-mediated excitation of VTA dopaminergic neurons known to regulate motivated feeding behaviours.

It is clear that ghrelin robustly increases the excitability of VTA dopamine neurons (Abizaid et al., 2006b; Jerlhag et al., 2007; Jerlhag, 2008; Kalafateli et al., 2018). It has been proposed that ghrelin encourages the activation of VTA dopaminergic neurons via two main mechanisms; directly by activating excitatory G-protein-coupled GHSRs ($G\alpha_q$) located on their perikarya and indirectly by increasing excitatory and decreasing inhibitory inputs and tone onto them (Abizaid et al., 2006b). Its direct capacity to excite VTA dopamine neurons is well understood and has been demonstrated electrophysiologically upon bath application of ghrelin

(Abizaid et al., 2006b). By contrast, the ability of ghrelin to indirectly activate these neurons by altering synaptic transmission onto them has only been explored in rodents treated peripherally with ghrelin (Abizaid et al., 2006b).

Nonetheless, ghrelin-induced activation of VTA dopaminergic neurons via either of these mechanisms triggers intracellular events (high intracellular calcium and enhanced production of DAG) known to promote 2-AG synthesis and release (Wang and Lupica, 2014; Yin et al., 2014; Edwards and Abizaid, 2016). In agreement with this, we have shown that VTA 2-AG levels are significantly lower in rodents with dysfunctional GHSRs relative to WT (Chapter 2). Appropriately, DAGL- α , the enzyme that produces 2-AG, is highly expressed on dendrites of VTA dopamine neurons and heightened endocannabinoid release is observed in response to increases in the activity of dopaminergic neurons within the VTA (Matyas et al., 2008; Riegel and Lupica, 2004). Together these data indicate that ghrelin-mediated excitation of VTA dopaminergic neurons likely encourages enhanced 2-AG tone and support the possible involvement of the endocannabinoid system in mediating the effects of ghrelin.

The mechanism by which endocannabinoids influence the excitability of VTA dopaminergic neurons is not clearly understood. It is largely accepted that CB-1Rs are not found on the cell bodies of VTA dopaminergic neurons (Mackie, 2005; Mátyás et al., 2008; Fitzgerald et al., 2012; Han et al., 2017); although some argue that CB-1Rs may be weakly expressed by VTA dopamine neurons (Hernandez et al., 2000; Wenger et al., 2003). CB-1Rs, are however, irrefutably expressed on presynaptic glutamatergic and GABAergic afferent terminals innervating the VTA (Mackie, 2005; Mátyás et al., 2008). These CB-1R-positive glutamatergic and GABAergic terminals contact and regulate neurotransmission onto both dopaminergic and non-dopaminergic neurons of the VTA (Szabo et al., 2002; Melis et al., 2004b, 2004a; Mackie, 2005; Mátyás et al., 2008; Oleson and Cheer, 2012; Merrill et al., 2015; Friend et al., 2017; Han et al., 2017). Consistent with this, CB-1R agonists have been shown to inhibit both excitatory and inhibitory tone onto VTA dopaminergic neurons (Herkenham et al., 1991; Szabo et al.,

2002; Melis et al., 2004b, 2004a; Mackie, 2005; Oleson and Cheer, 2012; Merrill et al., 2015; Friend et al., 2017; Han et al., 2017).

Here we conducted a series of electrophysiology experiments to elucidate where and how CB-1R signaling may modulate the capacity of ghrelin to increase the neuronal excitability of VTA dopaminergic neurons. Specifically, we first examined whether CB-1R antagonism would prevent the capacity of ghrelin to stimulate VTA dopaminergic neurons (i.e. depolarize and enhance action potential firing) and whether it could do so directly at the level of the dopamine neuron. To this end, we conducted current clamp recordings from fluorescent VTA dopaminergic neurons in *Th^{cre};L10-Egfp* mice, which express GFP in cells that naturally express tyrosine hydroxylase (a marker for catecholamine producing cells), and examined neuronal excitability changes induced by the bath application of ghrelin alone, when applied in the presence of CB-1R antagonists (rimonabant and AM-251), and when applied in the presence of CB-1R antagonists and tetrodotoxin (TTX). Since ghrelin also excites dopaminergic neurons by increasing excitatory and decreasing inhibitory tone onto these neurons when it is administered prior to animal sacrifice (Abizaid et al., 2006b), we tested the capacity of ghrelin to do so upon bath application. Accordingly, in voltage clamp experiments, we examined if bath application of ghrelin modulated the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) in dopaminergic neurons of the VTA. To assess the putative involvement of CB-1R signaling in ghrelin-induced synaptic transmission we also tested whether CB-1R antagonism could attenuate ghrelin-induced changes in synaptic transmission onto dopaminergic neurons of the VTA.

4.2 General methods

4.2.1 Animals

The *Th^{cre};L10-Egfp* mice that were used in electrophysiological experiments were generated by crossing heterozygous *Th^{cre}* mice (Jackson Laboratories, Bay Harbor, ME; JAX ID: 008601), a line that expresses cre recombinase under the tyrosine hydroxylase promoter, to homozygous *Egfp-L10a* mice, a line engineered with a floxed transcriptional stop cassette in front of a ribosomal protein L10-EGFP reporter construct (Jackson Laboratories; Jax ID: 024750). Approximately 50% of the progeny (*Th^{cre};L10-Egfp* mice) from this cross demonstrated constitutive expression of the *Egfp::L10A* fusion gene uniquely in neurons that express TH due to cre-mediated excision of the stop cassette. These mice facilitated the identification of putative dopamine neurons within the VTA when VTA-containing brain slices were illuminated with light of the appropriate wavelength (i.e. 395–488 nm). All transgenic lines that were used to generate the *Th^{cre};L10-Egfp* mouse line were bred on a C57BL/6 background. *Th^{cre};L10-Egfp* mice used for electrophysiology recordings were between 3–20 weeks old.

4.2.2 Immunohistochemistry

Male *Th^{cre};L10-Egfp* mice (~16 weeks old) were anesthetized with an intraperitoneal injection of urethane (1.6 g/kg) and were transcardially perfused with cold (4°C) isotonic saline followed by a 10% neutral buffered formalin (4°C) fixative solution. Following fixation, mice were decapitated and the brain was extracted from the skull. Brains were post-fixed (10% neutral buffered formalin) for a further 24 hours at 4°C and then transferred to 0.01 M phosphate-buffered saline (PBS) cyroprotectant solution containing 20% sucrose and 0.05% sodium azide (weight/volume) for twenty-four hours at 4°C. Brains were blocked, mounted, and sliced into 30 µm-thick coronal-plane sections using a freezing microtome (Leica SM2000R, Nussloch, Germany). Sections containing the VTA were stored in an antifreeze solution

containing 50% formalin, 30% ethylene glycol, and 20% glycerol until immunohistochemical staining.

Immunohistochemical studies were conducted under gentle agitation using VTA-containing brain sections. In brief, sections were rinsed with PBS six times for five minutes to wash away antifreeze solution. Sections were then incubated in a PBS blocking solution containing 3% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 0.25% Triton X-100, and 0.05% sodium azide for two hours at 21°C. Mouse anti-TH primary antibody (1:2000, Millipore, Burlington, MA) was added to the blocking solution and the sections were left to incubate overnight at 21°C. Sections were washed with PBS six times for five minutes before Cy-3 donkey anti-mouse secondary antibody (1:500, Jackson ImmunoResearch Laboratories, Inc.) was diluted in a PBS solution containing 3% NDS and 0.25% Triton X-100 and applied to brain sections. The brain sections were incubated in the secondary antibody for two hours at 21°C before being rinsed (three 10-minute PBS washes). VTA-containing brain sections were mounted on SuperFrost plus glass microscope slides (Fisher Scientific, Pittsburgh, PA) and coverslipped with ProLong Gold antifade reagent containing DAPI (Fisher Scientific). Confocal photomicrographs of VTA-containing slices were taken using a Nikon C2 confocal microscope (Nikon Instruments Inc., Mississauga, ON) at 10X magnification and were processed using NIS Elements software (Nikon). Native GFP fluorescence and TH (labelled with Cy-3) immunoreactivity were imaged with 488 nm and 561 nm excitation lasers, respectively.

4.2.3 Slice preparation

All procedures described herein were approved by the Carleton University Animal Care Committee and strictly followed the guidelines laid out by the Canadian Council on Animal Care. *Th^{cre};L10-Egfp* mice were decapitated and their brains were rapidly dissected out and immersed into cold (2–4°C), carbogenated (95% O₂, 5% CO₂) slicing solution (297–302 mOsm/L, pH 7.4) containing (in mM): 118 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 5 MgCl₂, 10 D-glucose, 26

NaHCO₃, and 0.5 CaCl₂. Brains were blocked and mounted on a stage submerged in slicing solution to facilitate either coronal or horizontal slicing on a vibratome (Leica VT 1000S, Leica Biosystems). Coronal (250 µm) or horizontal slices (200 µm) containing the VTA were cut and transferred to a warm (34°C), carbogenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) bath solution (297–302 mOsm/L, pH 7.4) containing (in mM): 124 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 10 D-glucose, 26 NaHCO₃, and 2.5 CaCl₂. Slices were incubated for 10 minutes at 34°C and then at room temperature (21–23°C) for a further 50 minutes to ensure recovery. Slices were maintained at room temperature (23°C) until used in patch clamp experiments.

4.2.4 Patch-clamp recordings

Th^{cre};L10-Egfp brain slices were placed in a slice chamber of a fixed stage (Gibraltar, ThorLabs, Newton, NJ) that was continuously superfused (2–2.5 ml/min, Master Flex CL Cole Palmer pump, Vernon Hills, IL) with carbogenated bath solution. This solution was warmed by a temperature controller (Warner Instrument Corporation, Hamden, CT) such that it entered the recording chamber between 32–34°C. A homemade platinum and nylon fiber harp was used to keep slices immobilized during recordings. Slices were illuminated with a Nikon power supply and visualized with an upright Eclipse E600FN microscope (Nikon) affixed with an Axio iCM1 camera (Zeiss, Oberkochen, Germany) that sits on a microscope translator (Gibraltar). Filamented borosilicate glass patch pipettes (OD: 1.5mm, ID:0.86mm, Sutter Instruments, San Francisco, CA), were pulled on a P-1000 Flaming/Brown micropipette puller (Sutter Instruments) to yield pipettes that had resistances between 5–8 MΩ when backfilled with a potassium gluconate based internal solution (285–288 mOsm/L, pH 7.24) containing (in mM): 120 K-gluconate, 10 KCl, 10 HEPES, 1 MgCl₂, 1 EGTA, 4 MgATP, 0.5 NaGTP, and 10 phosphocreatine. This potassium-based solution was used for all current clamp recordings and voltage clamp recordings of glutamatergic events held at –60 mV. Pipettes were filled with a cesium methanesulfonate based internal solution (285–288 mOsm/L, pH 7.24) containing (in mM): 125 CsMs, 11 KCl, 10 HEPES, 1 CaCl₂, 1 EGTA, 4 MgATP, and 0.5 NaGTP for IPSC voltage clamp experiments. Cells were held at –5mV for all IPSC recordings. Pipettes were affixed to a

MP-285A motorized 3-axis micromanipulator (Sutter Instruments) to facilitate approach of individual cells. The microscope, camera, micromanipulator, and Gibraltar were supported on a clean bench isolation table top supported by a Gimbal piston-air vibration isolation system (TMC, Peabody, MA) to minimize vibrational disruptions. A high magnification 60X water immersion objective lens was used to identify EGFP fluorescent cells within the VTA under epifluorescence illumination. Whole-cell patch-clamp recordings were made with pipettes connected to a headstage of an Axopatch 200B amplifier (Axon instruments) filtered at 2 kHz. The presence of a well-described hyperpolarizing cation current (I_h), characteristic of VTA dopaminergic neurons, was used to confirm that recorded green fluorescent cells were likely dopaminergic neurons (Johnson and North, 1992).

4.2.5 Drug applications

All drugs used in electrophysiology experiments were initially prepared as concentrated stock solutions and stored at -20°C until required. Rimonabant hydrochloride (Sigma Aldrich) and AM-251 (Tocris Biosciences) were dissolved in DMSO, acylated ghrelin (Tocris Biosciences) was dissolved in 0.9% isotonic saline and tetrodotoxin (Alomone labs) was dissolved in deionized distilled water. Drugs were diluted to their final concentrations in bath recording solution immediately before use. The final concentration of DMSO in CB-1R antagonist solutions never exceeded 0.1%.

For all experiments, a 15-minute baseline period, in which cells were continuously superfused with bath solution, was recorded to ensure cell stabilization before drug treatments were applied. Similarly, all cells were minimally given a 15-minute washout period after drug treatment completion to examine if drug induced effects were reversible. When ghrelin was applied alone it was perfused for a minimum of 3 minutes. For experiments investigating the combined effect of ghrelin and CB-1R antagonism, the slice was pretreated with 5 μM rimonabant or 2 μM AM-251 for 10 minutes prior to ghrelin application and was left on during and for 10 minutes after ghrelin application to ensure adequate CB-1R antagonism. For

experiments that involved tetrodotoxin, it was applied at the beginning of the 15-minute aCSF bath solution baseline period and remained on for all drug applications in addition to the washout period.

4.2.6 Resting membrane potential and action potential analysis

Resting membrane potential and action potential continuous trace recordings were obtained in current clamp without injection of current, except when cells were over (action potential frequency of 4 Hz or greater) or under active (very low resting membrane potential). In these instances, a small amount of negative or positive current was injected to prevent cell fatigue or to bring these cells closer to their firing thresholds, respectively.

The membrane potential of cells was sampled every second using Clampfit 10.7 (Axon Instruments, San Jose, CA) and averaged into 30 second bins. Resting membrane potential changes (Δ RMP) of VTA dopaminergic neurons relative to a 5-minute baseline period (immediately before ghrelin application) was calculated for each current clamp recording. A time course of the Δ RMP across cells was graphed to facilitate examination of drug-induced changes in resting membrane potential across time. In addition, representative Δ RMP means (across cells) were calculated and graphed for baseline, rimonabant, ghrelin, rimonabant & ghrelin, and washout periods to summarize treatment-induced changes in resting membrane potential.

An action potential template was created in Clampfit 10.7 (Axon Instruments) to detect the number of action potential events that exceed 0 mV in each of our current clamp recordings. The number of action potentials were organized into 30 second bins and action potential frequencies were calculated. A time course of action potential firing frequencies across cells was likewise graphed across time to examine drug-induced changes in firing rate of these VTA dopamine neurons. Once again, representative action potential frequency means were calculated and graphed for each treatment condition.

4.2.7 Spontaneous postsynaptic currents

sEPSCs and sIPSCs appeared as downward and upward deflections in voltage clamped continuous recordings, respectively. sEPSCs and sIPSCs were recorded in separate sets of cells. Mini Analysis (Synaptosoft, Decatur, GA) was used to detect the number of sEPSCs and sIPSCs present in voltage clamp recordings. The number of events were placed into 30 second bins and event frequencies were calculated. sEPSC and sIPSC frequencies were averaged across cells and graphed across time to facilitate detection of treatment-induced changes in excitatory and inhibitory tone onto dopaminergic neurons of the VTA.

4.2.8 Data and statistical analyses

Data from both voltage and current clamp experiments were collected and integrated using a Digidata 1322A (Molecular Devices) digitizer connected to a computer running Clampex 10.7 software (Axon Instruments). Current and voltage data were analyzed using Clampfit 10.7 (Axon Instruments) and Mini Analysis (Synaptosoft), respectively. Sample traces were created with Origin 2018 (OriginLab, Northampton, MA). Statistics and graphs were produced using Prism 7 (GraphPad, San Diego, CA). Results were represented as the mean $\pm$ SEM, with mean differences considered statistically significant at $p < .05$ unless otherwise stated.

Action potential frequency, Δ RMP, and sEPSC and sIPSCs frequency means were calculated for each treatment condition. For ghrelin only experiments, means were calculated right before ghrelin administration (baseline), at the peak ghrelin effect (ghrelin), and 10 minutes after ghrelin removal (washout). The same methodology was adopted for experiments when ghrelin was applied in the presence of TTX and/or CB-1R antagonists (i.e. baseline, ghrelin, and washout measurements were made in the presence of TTX and/or CB-1R antagonists). Statistical assumptions were tested (i.e. normality and sphericity) and appropriate corrections were made (when required) before treatment means were compared via repeated measures one-way ANOVAs. Tukey's post hoc tests were run when appropriate to detect significant differences in these measurements between treatments.

4.3 Results

4.3.1 Ghrelin depolarizes and increases action potential firing of VTA dopamine neurons

Whole cell recordings were obtained from GFP-expressing dopaminergic neurons within the lateral VTA in between the medial lemniscus and the interfascicular nucleus raphe as a majority of GFP-expressing neurons within this region likewise demonstrated TH-immunoreactivity upon immunohistochemical analysis of VTA-containing brain slices from *Th^{cre};L10-Egfp* mice (Figure 4.1A–B). Bath application of 500 nM ghrelin reversibly depolarized approximately 65% of VTA dopaminergic neurons (9 of 14) recorded ($F_{(2,16)} = 11.66$, $p = .0008$, partial $\eta^2 = .593$) (Figure 4.1C*-iii*). In ghrelin-responsive cells, ghrelin elicited a depolarization of almost 4 mV relative to baseline ($p = .001$), an effect that washed out within 10 minutes ($p = .0042$). This ghrelin-induced depolarization led to a corresponding significant increase in action potential firing in a majority of ghrelin-responsive cells ($F_{(2,14)} = 20.37$, $p < .0001$, partial $\eta^2 = .744$) (Figure 4.1C*i*, C*iv-v*). Ghrelin increased action potential firing of VTA dopaminergic neurons approximately 5-fold relative to baseline ($p < .0001$), an effect that slowly washed out ($p = .0042$) following ghrelin removal.

Figure 4.1: Ghrelin-mediated excitation of VTA dopaminergic neurons persists in the presence of CB-1R antagonists. (A) Confocal photomicrograph of the localization of native GFP and TH immunoreactivity in the VTA of *Thcre;L10-Egfp*. (B) Low magnification brightfield and (Bii) epifluorescent microscopy guides whole-cell patch clamp recordings of GFP-expressing dopaminergic neurons within the VTA. (C) Sample trace of ghrelin-induced changes in dopaminergic neuron excitability. (Cii) Time course of the change in resting membrane potential (Δ RMP) relative to baseline; (Ciii) summarized before (Con), during (Ghr), and following ghrelin washout (Wash). (Civ) Time course of action potential firing frequencies; (Cv) summarized before, during, and following ghrelin washout. Sample traces demonstrating the inability of rimonabant (D) and AM-251 (E) to block ghrelin-induced excitation of VTA dopamine neurons. (Dii, Eii) Time courses of Δ RMP relative to rimonabant or AM-251 baseline periods; (Diii, Eiii) summarized before, during, and following ghrelin washout. (Div, Eiv) Time courses of action potential firing frequencies; (Dv, Ev) summarized before, during, and following ghrelin washout. IF, interpeduncular nucleus; IPN, interpeduncular nucleus; ml, medial lemniscus; SN, substantia nigra; VTA, ventral tegmental area. All values are represented as mean $\pm$ SEM. *, $p < .05$; **, $p < .01$; ****, $p < .0001$.

4.3.2 Ghrelin-mediated excitation of VTA dopaminergic neurons persists in the presence of CB-1R antagonism

We next determined if CB-1R signaling contributed to the robust capacity of ghrelin to stimulate dopaminergic neurons within the VTA. The ability of ghrelin to depolarize VTA dopamine neurons was not blocked by pretreating and persistently applying rimonabant (5 out of 7) ($F_{(2,8)} = 4.328$, $p = .053$, partial $\eta^2 = .520$) (Figure 4.1Di-iii) or AM-251 (5 out of 12) ($F_{(2,8)} = 9.218$, $p = .0084$, partial $\eta^2 = .891$) (Figure 4.1Ei-iii) CB-1R antagonists. Ghrelin significantly depolarized these neurons compared to rimonabant ($p = .063$) and AM-251 ($p = .0069$) baseline levels. The depolarizing effect of ghrelin failed to washout in the presence of rimonabant ($p = .943$) but tended to washout in AM-251 ($p = .076$). Although the ghrelin-induced depolarization did not lead to statistically significant increases in action potential firing of VTA dopaminergic neurons in the presence of rimonabant ($F_{(2,4)} = 3.191$, $p = .148$, partial $\eta^2 = .615$) (Figure 4.1Di, Div-v) or AM-251 ($F_{(1,3)} = 1.458$, $p = .314$, partial $\eta^2 = .697$) (Figure 4.1Ei, Eiv-v) (largely due to inadequate power associated with small n), visual inspection of these data suggested that ghrelin, in the presence of either antagonist, increased action potential firing of these neurons to a degree comparable to ghrelin administered alone.

4.3.3 Ghrelin does not require CB-1R signaling to directly depolarize VTA dopaminergic neurons

Consistent with a direct, CB-1R independent postsynaptic effect, the capacity of ghrelin to depolarize VTA dopamine neurons persisted in the presence of 500 nM TTX and 5 μ M rimonabant (6 out of 8) ($F_{(2,10)} = 12.48$, $p = .0019$, partial $\eta^2 = .714$) (Figure 4.2A-B). Despite CB-1R antagonism and blocked activity dependent synaptic transmission, ghrelin elicited a depolarization of almost 4 mV relative to baseline ($p = .002$), an effect comparable to the effect of ghrelin administration in the absence of TTX (Figure 4.1C). This effect slowly washed out following the removal of ghrelin ($p = .011$).

Figure 4.2: Ghrelin directly activates VTA dopaminergic neurons independent of CB-1R signaling. **(A)** Sample trace of the Δ RMP induced by the application of ghrelin in the presence of TTX and rimonabant. **(Bi)** Time course of Δ RMP relative to rimonabant baseline; **(Bii)** summarized before (Rim), during (Ghr), and following ghrelin washout (Wash in rimonabant). **, $p < .001$.

4.3.4 Ghrelin heterogeneously modulates GABA but increases glutamatergic tone onto VTA dopaminergic neurons

In addition to directly stimulating VTA dopaminergic neurons, ghrelin has also shown the capacity to modulate synaptic transmission onto VTA dopamine neurons to increase their excitability (Abizaid et al., 2006b). Given this and that CB-1R antagonism was not observed to impact the actions of ghrelin at the level of the dopamine neuron, we next explored the possibility that ghrelin and endocannabinoid systems may collaboratively modulate synaptic transmission onto VTA dopamine neurons to regulate their activity. Since the capacity of ghrelin to influence synaptic transmission onto VTA dopaminergic neurons had only been demonstrated with peripheral injection of ghrelin (Abizaid et al., 2006b), we first tested if ghrelin could likewise do so when directly applied to slices containing the VTA.

Bath application of 500 nM ghrelin altered GABA tone onto approximately 60% of VTA dopamine neurons recorded (10 out of 17) (Figure 4.3). Interestingly, ghrelin bi-directionally modulated the frequency of sIPSCs onto these VTA dopaminergic neurons; significantly decreasing (4 out of 10) ($F_{(2,6)} = 7.647$, $p = .0224$, partial $\eta^2 = .718$) (Figure 4.3A) it in some cases, while increasing (6 out of 10) ($F_{(2,10)} = 18.83$, $p = .0004$, partial $\eta^2 = .792$) (Figure 4.3B) it in others. In instances where ghrelin suppressed sIPSC frequency, ghrelin reduced sIPSC frequency by approximately 40% relative to baseline ($p = .019$) (Figure 4.3Aii-iii). In instances where ghrelin heightened sIPSC frequency, ghrelin increased the sIPSC frequency almost 2-fold relative to baseline ($p = .0004$) (Figure 4.3Bii-iii). In both cases, sIPSC frequency tended to rebound toward baseline levels following ghrelin removal (i.e. no significant difference between baseline and washout conditions) ($p > .05$).

In contrast to its heterogeneous regulation of GABA tone, ghrelin solely and robustly increased the frequency of sEPSCs onto approximately 60% of VTA dopamine neurons recorded (7 out of 12) ($F_{(2,6)} = 5.217$, $p = .0234$, partial $\eta^2 = .465$) (Figure 4.4Ai-iii). On average ghrelin increased sEPSC frequency 3-fold relative to baseline ($p = .0297$), an effect that dissipated with ghrelin washout ($p = .057$).

Figure 4.3: Heterogeneous effects of ghrelin on GABAergic inputs to dopaminergic VTA neurons. Sample traces of ghrelin (**Ai**) suppressing, (**Bi**) potentiating, (**Ci**) or having no effect on the frequency of sIPSCs. (**Aii**, **Bii**, **Cii**) Time courses of sIPSC frequencies; (**Aiii**, **Biii**, **Ciii**) summarized before, during, and following ghrelin washout. All values are represented as mean $\pm$ SEM. *, $p < .05$; **, $p < .01$; ***, $p < .001$.

4.3.5 CB-1R antagonism blocks ghrelin-mediated increase in glutamatergic tone onto VTA dopaminergic neurons

Given that ghrelin had heterogeneous effects on GABA synaptic transmission but a clear unidirectional capacity in augmenting excitatory tone on VTA dopaminergic neurons, we next examined if CB-1R signaling was required for ghrelin-mediated increases in glutamatergic tone onto these neurons. Interestingly, CB-1R antagonism significantly blocked ghrelin-mediated increases in sEPSC frequency in a majority of cells recorded (6 out of 7) ($F_{(2,10)} = 2.187$, $p = .163$, partial $\eta^2 = .304$) (Figure 4.4Bi-iii).

Figure 4.4: CB-1R antagonism blocks ghrelin-mediated increases in glutamatergic tone onto VTA dopaminergic neurons. **(Ai)** Sample trace, **(Aii)** time course, and **(Aiii)** summary of sEPSC frequency changes induced by ghrelin. **(Bi)** Sample trace, **(Bii)** time course, and **(Biii)** sEPSC frequency summarized before, during, and following ghrelin washout in AM-251. All values are represented as mean $\pm$ SEM. *, $p < .05$.

4.4 Discussion

The present results reaffirm the robust capacity of ghrelin to acutely increase the neuronal excitability of VTA dopaminergic neurons and have clarified where and the extent to which the endocannabinoid system appears to contribute. Specifically, we demonstrated that ghrelin potently depolarizes and increases action potential firing of VTA dopamine neurons in a CB-1R antagonist insensitive manner. Moreover, we determined that ghrelin is able to depolarize these neurons directly and independently of CB-1R signaling. In addition to evaluating the direct excitatory action that ghrelin has on VTA dopaminergic neurons we also probed its capacity to modulate synaptic transmission onto VTA dopamine neurons upon bath application. While ghrelin elicited heterogeneous effects with respect to GABA neurotransmission onto VTA dopamine neurons, it potently increased the frequency of glutamate release onto these neurons indicating that ghrelin also indirectly stimulates VTA dopaminergic neurons by increasing excitatory tone onto them. Interestingly, CB-1R antagonism prevented ghrelin-induced increases in excitatory tone in a majority of cells recorded suggesting that endocannabinoids likely mediate the capacity of ghrelin to indirectly promote the activation of VTA dopaminergic neurons. Together, these data indicate that while endocannabinoid signaling is not required for ghrelin to directly activate VTA dopaminergic neurons, it is required for ghrelin to indirectly promote their activity by enhancing excitatory tone onto these neurons.

The capacity of ghrelin to reversibly depolarize and increase action potential firing of VTA dopaminergic neurons, demonstrated herein, is consistent with the work of Abizaid et al. (2006b), who first demonstrated that ghrelin increases action potential firing of VTA dopaminergic neurons via activation of GHSRs. These data are also congruent with numerous rodent studies that report increased dopamine turnover and release following intra-VTA ghrelin administration (Abizaid et al., 2006b; Jerlhag et al., 2007, 2010; Kalafateli et al., 2018). Interestingly, we found that ghrelin increased the frequency of action potentials to a much greater extent relative to baseline (almost 5-fold) compared to those reported previously (~

1.5-fold) (Abizaid et al., 2006b). The apparent disparity in the magnitude of ghrelin-induced effects may predominantly be driven by the higher basal action potential frequencies observed by Abizaid and colleagues. Accordingly, although our change from baseline was more pronounced, the raw ghrelin-induced action potential frequencies reported by Abizaid et al. (2006b) were almost double those presented here. This could be due to differences in the holding current used during recordings. Although herein we predominantly recorded cells without injecting current, when cells were over active (action potential frequency of 4 Hz or greater) or under active (very low resting membrane potential) positive or negative current was injected to prevent cell fatigue or to bring cells closer to their action potential firing thresholds. Therefore it is possible that cells in this study were clamped at a lower potential compared to that used by Abizaid et al. (not reported) (Abizaid et al., 2006b). Alternatively, this discrepancy may be driven by the age of mice used for electrophysiological recordings as Abizaid and colleagues used much younger mice (2–3 weeks) than those used herein (3–20 weeks). In support of this, others report higher action potential firing frequencies of VTA dopaminergic neurons in young rodents (McCutcheon et al., 2012). Nevertheless, the time course of the initiation and washout of ghrelin-induced increases in action potential firing are compatible with those reported by Abizaid et al. (2006b). Importantly, in addition to corroborating the known ability of ghrelin to increase action potentials firing in VTA dopamine neurons, we demonstrated that ghrelin, in most cases, is capable of doing so by depolarizing these neurons by only a few millivolts (2–4 mV).

Interestingly, we found that ghrelin-mediated excitation of VTA dopaminergic neurons persisted in the presence of two different cannabinoid receptor antagonists (i.e. rimonabant and AM-251). In the presence of these CB-1R antagonists, ghrelin significantly depolarized these neurons and tended to enhance action potential firing frequencies to a similar extent as when ghrelin was administered alone. These data suggest that ghrelin does not require CB-1R signaling to stimulate dopaminergic neurons of the VTA. This together with our findings that ghrelin maintained its capacity to depolarize VTA dopamine neurons in the presence of CB-1R antagonism and absence of activity-driven synaptic transmission (i.e. TTX), highlight that ghrelin

is capable of directly stimulating the perikarya of VTA dopaminergic neurons via CB-1R independent processes. These data are in line with the presence of excitatory GHSRs (Abizaid et al., 2006b) and absence of inhibitory CB-1Rs (Mackie, 2005; Mátyás et al., 2008; Fitzgerald et al., 2012; Han et al., 2017) on dopaminergic neuron perikarya within the VTA. By contrast and at odds with our findings, Abizaid and colleagues (2006b) demonstrated a severely hindered capacity of ghrelin to increase action potential firing of dopamine neurons in the presence of AMPA and NMDA receptor antagonists (CNQX and AP-5, respectively) suggesting that ghrelin normally induces and requires excitatory synaptic activity to fully stimulate VTA dopaminergic neurons (Abizaid et al., 2006b). Numerous factors may help explain these apparent disparate findings. First, Abizaid and colleagues (2006b) solely identified putative dopamine neurons as those that possessed a hyperpolarizing cation current (I_h) proposed to be characteristic of a majority of VTA dopaminergic neurons (Johnson and North, 1992); whereas, we used *Th^{cre};L10-Egfp* mice and the presence of this current to mitigate our changes of recording from non-dopaminergic neurons within the VTA. Investigations into the validity of using the hyperpolarizing cation current as a marker of dopamine neurons within the VTA argue that the absence of this current reliably predicts that a VTA neuron is not dopaminergic but its presence does not guarantee a neuron is dopaminergic (Margolis et al., 2006). Therefore, it may be that the direct ability of ghrelin to excite VTA dopaminergic neurons in the absence of excitatory tone, observed herein, was diluted by the inclusion of non-dopaminergic neurons in studies conducted by Abizaid and colleagues. Second, Abizaid and colleagues investigated the capacity of ghrelin to enhance action potential firing in VTA dopaminergic neurons under conditions of blocked excitatory tone but they did not simultaneously inhibit GABAergic tone; therefore, it is possible that the direct depolarizing actions of ghrelin were somewhat antagonized by this unbalanced inhibitory tone (Abizaid et al., 2006b). Third, Abizaid and colleagues pooled results from all recorded neurons which likely diluted their capacity to detect the direct excitatory action of ghrelin on dopaminergic neurons of the VTA as only 60% of these neurons are thought to express GHSRs (Abizaid et al., 2006b). The aforementioned arguments offer putative explanations of why we were able to examine a clear and direct action of ghrelin on VTA dopamine neurons in the absence of activity-dependent synaptic transmission while Abizaid

and colleagues did not when they blocked glutamate receptors. That said, the fact that Abizaid and colleagues were able to blunt the capacity of ghrelin to excite VTA dopamine neurons with glutamate receptor antagonists clearly highlights that ghrelin also promotes excitatory neurotransmission onto VTA dopaminergic neurons to increase their neuronal excitability (Abizaid et al., 2006b).

In line with this and experiments demonstrating that peripheral ghrelin increases excitatory tone onto dopaminergic neurons of the VTA (Abizaid et al., 2006b), we found that ghrelin significantly enhanced glutamate release onto VTA dopaminergic neurons when applied to slices containing the VTA. Interestingly, we determined that this ghrelin-mediated increase in glutamatergic tone onto VTA dopaminergic neurons was blocked by CB-1R antagonism suggesting that endocannabinoids normally mediate the indirect excitatory effect of ghrelin onto VTA dopamine neurons. Although one could argue that the 6 cells (out of 7), which did not increase their sEPSC frequency in the presence of the CB-1R antagonist, were ghrelin-insensitive cells, we do not believe that this was the case as ghrelin increased sEPSCs in approximately 60% of VTA dopaminergic neurons when it was administered alone. This proportion is consistent with the ghrelin-induced effects observed across all electrophysiological studies conducted (48 out of 77 cells). Future studies will address this concern by confirming that cells demonstrate a ghrelin effect before testing whether CB-1R antagonism can prevent subsequent ghrelin-induced increases in sEPSCs. The neurocircuitry underlying this effect has yet to be elucidated; however, one possible explanation is that CB-1R-positive GABAergic terminals make axo-axonic synapses with and tonically inhibit glutamatergic neurons that regulate VTA dopaminergic neurons. Provided this is the case, when applied alone, ghrelin would directly activate VTA dopamine neurons and increase 2-AG production and release. Ghrelin-induced endocannabinoids would then act retrogradely to suppress GABA release onto the tonically inhibited glutamate neurons (i.e. disinhibit them) allowing them to increase glutamatergic tone onto the dopamine neurons of the VTA (i.e. ghrelin-induced increases in sEPSCs). In the presence of a CB-1R antagonist, the ghrelin-induced endocannabinoid tone would not disinhibit glutamate neurons thus eliminating the capacity of

ghrelin to increase sEPSCs in dopaminergic neurons of the VTA. While the aforementioned theory suggests that ghrelin acts solely at GHSRs on VTA dopaminergic to activate this neurocircuit and increase sEPSCs onto VTA dopamine neurons, one must also consider that presynaptic activation of GHSRs on glutamatergic terminals may likewise contribute to the enhanced sEPSCs observed upon ghrelin application. Consistent with this, ghrelin acts presynaptically to modulate synaptic tone in other feeding related brain regions (e.g. HYP) (Cowley et al., 2003; Diano et al., 2006; Yang et al., 2011; Cabral et al., 2012, 2016) and GHSR immunoreactivity surrounding the cell body and dendrites of VTA dopamine neurons is consistent with a presynaptic localization of GHSRs (Abizaid et al., 2006b). While the above theories are consistent with our current electrophysiological data and location of ghrelin and endocannabinoid signaling machinery within the VTA, future studies are needed to test their validity. For example, experiments examining whether ghrelin induces EPSCs in the presence of GABA_A receptor antagonists or in the presence of TTX will be helpful in determining if this CB-1R dependent ghrelin effect is in fact mediated as stated above (via an axo-axonic GABA synapse). In addition, examining whether ghrelin-induces sEPSC onto VTA dopamine neurons with a patch pipette filled when a DAGL- α inhibitor and/or a calcium chelator (conditions known to prevent endocannabinoid synthesis) will help elucidate whether VTA dopamine neurons are in fact the source of endocannabinoids and whether the capacity of ghrelin to increase sEPSCs predominantly relies on the direct activation of GHSRs on VTA dopamine neurons. These proposed future studies should help clarify how CB-1R signaling mediates the ability of ghrelin to indirectly promote the activity of VTA dopaminergic neurons by increasing excitatory tone onto them.

In contrast to reports demonstrating that peripheral ghrelin potently decreases the number of GABAergic inputs and reduces GABAergic tone onto VTA dopaminergic neurons (Abizaid et al., 2006b), we found that bath application of ghrelin heterogeneously modulated GABAergic release onto dopaminergic neurons of the VTA. These data suggest that although ghrelin clearly influences GABA release onto VTA dopamine neurons, its capacity to stimulate VTA dopamine neurons is not likely driven by its ability to inhibit GABAergic tone onto them.

This notion is supported by work demonstrating that the GABA_A receptor antagonist bicuculline does not enhance the capacity of ghrelin to increase the neuronal excitability of VTA dopaminergic neurons (i.e. frequency of action potentials) (Abizaid et al., 2006b). Given that the goal of these studies was to elucidate how CB-1R signaling might modulate ghrelin-mediated excitation of VTA dopaminergic neurons and that ghrelin heterogeneously modulated GABAergic neurotransmission onto VTA dopaminergic neurons, we did not interrogate how CB-1R signaling impacted ghrelin-induced GABA tone onto VTA dopamine neurons herein. Nevertheless, it is tempting to speculate that the ghrelin-induced suppression of inhibitory tone onto VTA dopamine may be endocannabinoid mediated. In support of this, ghrelin robustly activates VTA dopaminergic neurons, a process known to stimulate 2-AG production and release (Szabo et al., 2002; Melis et al., 2004a, 2004b; Riegel and Lurpica, 2004; Abizaid et al., 2006b; Mátyás et al., 2008; Oleson et al., 2012), and CB-1R-positive GABAergic terminals innervate VTA dopaminergic neurons (Mátyás et al., 2008). Accordingly, ghrelin-induced excitation of VTA dopamine neurons may promote endocannabinoid mediated retrograde inhibition of GABA release onto VTA dopamine neurons. In contrast, the ghrelin-mediated rise in sIPSCs observed in the rest of the ghrelin-sensitive cells could be due to the direct activation of excitatory GHSRs in GABAergic cells that innervate these dopaminergic neurons. Consistent with this, GHSRs are expressed in approximately 30% of GABAergic cells of the VTA (Abizaid et al., 2006b). Nevertheless, future studies similar in nature to those proposed to elucidate the interaction of ghrelin and endocannabinoid systems at glutamatergic terminals will be conducted to investigate if and how endocannabinoids modulate GABAergic tone onto VTA dopaminergic neurons of the VTA.

In summary, together our results demonstrate that while endocannabinoid signaling does not appear to be imperative for ghrelin to directly activate VTA dopaminergic neurons it is required for ghrelin to indirectly promote the probability of their activation by enhancing excitatory tone.

Chapter 5

Experimental considerations and implications of the interaction between ghrelin and endocannabinoid systems

5.1 General discussion

The present thesis set out to determine if ghrelin and endocannabinoid systems interact within the VTA and the nature and extent to which this interaction modulates motivated feeding behaviours. Accordingly, the experiments conducted and described throughout this thesis provide compelling molecular (Chapter 2), behavioural (Chapter 3), and electrophysiological (Chapter 4) evidence supporting an interaction between these systems within the VTA. Importantly, these studies have identified that CB-1R signaling is necessary for ghrelin to fully engage the appropriate neurocircuits (i.e. VTA dopaminergic neurons) within the VTA that drive motivated feeding behaviours and ultimately for the manifestation of these behaviours.

Our data confirm that ghrelin, like other metabolic hormones (i.e. leptin and insulin), modulates the activity of VTA dopamine neurons and influences feeding behaviours when infused within the VTA (Fulton et al., 2006; Hommel et al., 2006; Labouebe et al., 2013). More importantly, our findings indicate that endocannabinoid signaling contributes to these effects. At the cellular level, we have shown that CB-1R signaling is not required for ghrelin to directly activate VTA dopaminergic neurons but that it is required for ghrelin to indirectly promote their activity by enhancing excitatory tone onto them. These findings highlight a previously unrecognized and CB-1R dependent mechanism by which ghrelin regulates the activity of VTA dopamine neurons and provide a putative explanation for the attenuation of ghrelin-induced feeding behaviours that accompanied intra-VTA CB-1R antagonism.

5.1.1 Experimental considerations

It is important to note that this project focused on deducing whether endocannabinoid signaling mediated the excitatory effects of ghrelin on dopamine cells and motivated feeding behaviours. Although we were successful in this regard, we would like to outline the following considerations. First, we explored the interaction between ghrelin and endocannabinoids at excitatory inputs to VTA dopamine cells; however, it is also important to investigate their putative interaction at inhibitory inputs. Second, in addition to dopaminergic neurons, there are also non-dopaminergic cell groups that may mediate the effects of ghrelin in the VTA. We address these issues below.

In the present thesis we evaluated whether ghrelin and endocannabinoid systems interacted directly at the level of VTA dopaminergic neurons and whether CB-1R signaling mediated the capacity of ghrelin to increase excitatory tone onto these cells. Given that ghrelin heterogeneously modulated GABAergic tone onto VTA dopamine neurons, we did not prioritize assessing the role of CB-1R signaling in mediating this process. However, many factors suggest that CB-1R signaling may play a role in modulating ghrelin-induced GABAergic neurotransmission onto VTA dopaminergic cells. VTA dopamine neurons are strongly regulated by GABAergic inputs. Accordingly, GABAergic contacts comprise 50–80% of all synapses at VTA dopaminergic neurons (Bayer and Pickel, 1991; Edwards et al., 2017) and these GABA axon terminals are known to express CB-1Rs (Mátyás et al., 2008). We have shown that ghrelin directly activates VTA dopamine neurons and others have shown that activation of VTA dopamine neurons leads to endocannabinoid release (Lupica and Riegel, 2005). Therefore, ghrelin-mediated excitation of VTA dopamine neurons presumably encourages endocannabinoid release. This may lead to endocannabinoid-mediated suppression of GABA release at VTA dopamine neurons and ultimately to VTA dopamine neuron disinhibition. Our data demonstrating suppressed ghrelin-induced motivated feeding upon CB-1R antagonism (Chapter 3) and decreased sIPSC frequency upon ghrelin application are consistent with this model (Chapter 4). In contrast, our data demonstrating ghrelin-induced increases in sIPSC

frequency are more consistent with the direct activation of excitatory GHSR on GABAergic cells of the VTA. Future studies are needed to clarify if ghrelin preferentially increases or decreases GABA tone onto VTA dopamine neurons and if CB-1R signaling mediates these effects.

We demonstrated that CB-1R signaling is required for ghrelin-induced motivated feeding within the VTA and involved in ghrelin-mediated excitation of VTA dopamine neurons; however, it is possible that ghrelin and endocannabinoid systems also interact at other cells within the VTA. In addition to dopamine neurons, GABA (30–35%) and glutamate (5–45%) neuronal populations are also prominent within the VTA and contribute to the regulation of feeding behaviours when activated (Margolis et al., 2006; Nair-Roberts et al., 2008; Tan et al., 2012; van Zessen et al., 2012; Li et al., 2013; Creed et al., 2014; Root et al., 2014; Wang et al., 2015a; Zhang et al., 2015; Berrios et al., 2016; Yoo et al., 2016; Morales and Margolis, 2017; Root et al., 2018). For example, activation of VTA GABAergic neurons prevents reward-related motivated behaviours (e.g. feeding) both by directly antagonizing neighbouring VTA dopaminergic neurons (Tan et al., 2012; van Zessen et al., 2012; Creed et al., 2014) and through actions in efferent targets of the VTA (Creed et al., 2014). Given the excitatory nature of GHSRs (Yin et al., 2014) it is highly unlikely that ghrelin acts at GABAergic neurons to promote feeding behaviours. By contrast, activation of VTA glutamate neurons is positively reinforcing and induces motivated behaviours by increasing glutamate release onto VTA dopamine neurons as well as in brain regions innervated by the VTA (e.g. NA, VP) (Wang et al., 2015a; Yoo et al., 2016). It is therefore possible that ghrelin may stimulate VTA glutamatergic neurons within the VTA to promote motivated feeding behaviours via a process that is more reliant on CB-1R signaling. Consistent with this, CB-1Rs are expressed by VTA glutamatergic neurons (Han et al., 2017) and these neurons are likewise innervated by excitatory and inhibitory CB-1R-positive axon terminals (Mátyás et al., 2008). It is not known if GHSRs are expressed in VTA glutamatergic neurons; however, our data and that of others, demonstrate that ghrelin application enhances glutamate neurotransmission onto VTA dopaminergic neurons. Therefore, it is of interest to determine if ghrelin directly activates VTA glutamatergic neurons in patch clamp recordings and whether the actions of ghrelin are also CB-1R dependent.

5.1.2 Research extensions

We established that CB-1R signaling is involved in ghrelin-mediated excitation of VTA dopamine neurons and required for ghrelin-induced motivated feeding within the VTA; however, it is also of interest to determine if the endocannabinoid system similarly requires GHSR signaling to engage this neurocircuit and enhance motivated feeding behaviours. Our data demonstrating suppressed gene expression of important endocannabinoid system proteins and reduced 2-AG levels in the VTA of GHSR KO animals relative to WT animals, support this notion (Chapter 2). In line with this, others have shown that CB-1R agonists reliably initiate adipogenic and orexigenic signaling cascades and induce feeding in WT animals but fail to do so in GHSR KO animals (Lim et al., 2013). That said, it does not appear that ghrelin is unique in engaging the endocannabinoid system to modulate the activity of VTA dopamine neurons and associated feeding behaviours (Wang and Lupica, 2014). For example, feeding-related peptides, such as insulin and neurotensin, have been shown to reduce excitatory tone onto VTA dopamine neurons and dampen the activity of VTA dopamine neurons through endocannabinoid-mediated presynaptic inhibition of glutamate release (Kortleven et al., 2011, 2012; Labouebe et al., 2013; Schroeder and Leininger, 2018). Therefore, it seems that although intra-VTA CB-1R agonist administration enhances the activity of VTA dopamine neurons and promotes food intake, endocannabinoids do not indiscriminately promote the activity of these neurons to promote feeding. Nevertheless, future studies that evaluate whether intra-VTA administration of MAGL and FAAH inhibitors (i.e. raising endocannabinoid tone within the VTA) stimulates VTA dopaminergic neurons and/or promotes food intake in WT and GHSR KO animals, would clarify the natural role of endocannabinoids in eliciting these effects and their reliance on the ghrelin system.

There is significant overlapping expression of GHSRs and CB-1Rs throughout the brain, such as in the hippocampus (HIP), area postrema, and nucleus tractus solitarius. We thus postulate that a similar mechanism of GHSR action might be common in these regions. For example, GHSRs and CB-1Rs are highly expressed (Herkenham et al., 1991; Zigman et al., 2006; Mani et al., 2014) and contribute to synaptic plasticity processes in the HIP (Wilson and Nicoll,

2001; Diano et al., 2006; Isokawa, 2012; Cahill et al., 2014), a brain region that is increasingly gaining recognition for being involved in regulating feeding behaviours (Liu and Kanoski, 2018; Suarez et al., 2019). HIP dependent spatial and contextual memories seem to be important for remembering locations of food sources and other features of episodic memory that are associated with obtaining and consuming foods (Suarez et al., 2019). Recent evidence shows that infusion of ghrelin into the HIP stimulates motivated feeding behaviour and increases initiation of meals in response to food-related cues (Kanoski et al., 2013). While it is unknown if endocannabinoids mediate ghrelin-induced feeding within the HIP, based on our data, one could hypothesize that ghrelin produces these effects through the stimulation of the endocannabinoid system. Ultimately, the discovery and investigation of sites at which ghrelin and endocannabinoid systems interact will allow us to determine if endocannabinoids are required for GHSR-mediated effects in general, or if this is a feature that is only observed in specific brain regions like the VTA (Edwards and Abizaid, 2016).

5.2 Conclusions

Together these data provide compelling evidence that ghrelin and endocannabinoid systems interact within the VTA and that ghrelin requires CB-1R signaling to fully engage VTA dopaminergic neurons. Here we examined the capacity of ghrelin to induce motivated feeding behaviours; however, ghrelin also acts within the VTA to increase motivated behaviours to obtain rewards other than food, such as sex and drugs of abuse (Jerlhag et al., 2009, 2010; Prieto-Garcia et al., 2015; Hyland et al., 2018). Thus, given our data, we believe that endocannabinoids may, in the same manner, mediate the capacity of ghrelin to increase sexual motivation and drug seeking behaviours. The interaction between ghrelin and endocannabinoid systems could be exploited to develop combinatorial therapeutics to either increase or suppress motivated behaviours whilst minimizing adverse side effects.

Chapter 6

Appendix A: Supplemental figures, tables, and co-author permission statements

6.1 Supplementary figures

Supplementary figure 1: Simplified depiction of the major afferents and efferents of the mesocorticolimbic dopamine system. **AMG**, amygdala; **BNST**, bed nucleus of the stria terminalis; **HIP**, hippocampus; **LDT**, laterodorsal tegmental nucleus; **mPFC**, medial prefrontal cortex; **NA**, nucleus accumbens; **PPT**, pedunculo pontine nucleus; **RMTg**, rostromedial tegmental nucleus; **VP**, ventral pallidum; **VTA**, ventral tegmental area. Note image was taken from (Edwards and Abizaid, 2016).

Supplementary figure 2: Schematic representation of the putative independent (**A, B**) and collaborative (**C**) action of ghrelin and endocannabinoids within the VTA. (**A**) Ghrelin stimulates dopaminergic neurons of the VTA directly by binding GHSRs on their surface as well as indirectly by increasing excitatory and decreasing inhibitory inputs. (**B**) Endocannabinoids presumably stimulate VTA DA neurons by suppressing presynaptic GABA release to a greater extent than glutamate release. (**C**) Ghrelin-mediated activation of VTA DA neurons promotes 2-AG synthesis and release, which reduces GABAergic tone to further excite VTA DA neurons. Adapted image from (Edwards and Abizaid, 2016).

Supplementary figure 3: Experimental design for food intake and locomotor assessment. Rats received one of the following four treatments: rimonabant (0.5 µg) /ghrelin (1 µg), rimonabant (0.5 µg)/saline, vehicle/ghrelin (1 µg), or vehicle/saline via two separate 0.5 µl microinjections (infused over 2 minutes), spaced 30 minutes apart. Cumulative food intake and ambulatory activity were monitored 30, 60, 120, 240, and 360 minutes after the second microinfusion.

Supplementary figure 4: (A) Food-reward operant conditioning experimental timeline. **(B)** On test day, rats were randomly assigned to one of the four following treatments: rimobantant (0.5 µg) /ghrelin (1 µg), rimobantant (0.5 µg)/saline, vehicle/ghrelin (1 µg), or vehicle/saline. These combinatorial treatments were given via two separate 0.5 µl microinjections (infused over 2 minutes), spaced 30 minutes apart. Immediately following the second microinfusion rats were placed in the operant chamber for their PR test session. **(C)** The PR schedule used to assess feeding motivation. Rats had 30 minutes to obtain each reward. The amount of food rewards obtained before the animal timed out was defined as their breakpoint and was taken as an index of how motivated the rat was to work for food rewards.

6.2 Supplementary table

Table 1: RT-qPCR primer sequences

Name	Primer	Reference
β -actin-Forward	5'-TCATGAAGTGTGACGTTGACATCCG-3'	(Ni et al., 2006)
β -actin-Reverse	5'-CCTAGAAGCATTTGCGGTGCACGATG-3'	(Ni et al., 2006)
GAPDH-Forward	5'-AAGATGGTGAAGGTCGGTGT-3'	(Zhang et al., 2018)
GAPDH-Reverse	5'-CTTGCCGTGGTAGAGTCAT-3'	(Zhang et al., 2018)
DAGL- α -Forward	5'-GCCACAGAGCATCGCAACAG-3'	(Giannone et al., 2012)
DAGL- α -Reverse	5'-ACAGCAGCCGTCCTTCAGAG-3'	(Giannone et al., 2012)
FAAH-Forward	5'-ATGGACAGACGGCAGTGCAG-3'	(Porcella et al., 2002)
FAAH-Reverse	5'-GGCTTCAGCAGGAGGCTCAG-3'	(Porcella et al., 2002)
MAGL-Forward	5'-TAGCAGCTGCAGAGAGACCA-3'	(Duncan et al., 2008)
MAGL-Reverse	5'-GATGAGTGGGTCGGAGTTGT-3'	(Duncan et al., 2008)
NAPE-PLD-Forward	5'-AGATATGGACTCAAGAGTGAAGACTTC-3'	(Coria et al., 2014)
NAPE-PLD-Reverse	5'- TCCTCAAAGGCTTTGTCATCGG-3'	(Coria et al., 2014)

6.3 Co-author permission statements

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