It Takes Two to Tango: Investigating the Relationship between Ghrelin and Endocannabinoids within the Ventral Tegmental Area with Regards to Feeding

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Abstract

Ghrelin is a hormone that targets the brain to increase food intake and energy balance. Recent evidence suggests that ghrelin increases appetite in part by acting on growth hormone secretagogue receptors (GHSR) in the ventral tegmental area (VTA), a brain region associated with reward seeking behaviors. The ability of ghrelin to induce appetite is reminiscent of the appetite inducing effects of endogenous cannabinoids (CBs). Interestingly, ghrelin's ability to stimulate feeding within the hypothalamus is dependent on a functional CB system within this region. In the present thesis we hypothesized that ghrelin and CB systems work in tandem within the VTA to stimulate feeding. Here we showed that ghrelin significantly increased food intake ($p < .05$) when directly microinjected into the VTA of rats. Furthermore, we demonstrated that this corresponding increase in food intake depended on a functional endocannabinoid system as peripheral pre-treatment with a selective CB-1 receptor (CB-1R) antagonist (i.e. rimonabant) completely attenuated this increase in food intake to control rat levels. Furthermore, we also demonstrated *via* reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments that CB-1R mRNA expression is significantly lower in the VTA but enhanced in the prefrontal cortex (PFC) of GHSR knock-out (GHSR KO) relative to wild-type (WT) rats ($p < .05$). Together, these data provide evidence that ghrelin targets the VTA to increase food intake through a mechanism that requires a functional endocannabinoid system.

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Preface

The following thesis will begin with an introduction briefly highlighting the merit and importance of feeding related neurocircuitry research in combating the “obesity epidemic”. It will then transition into a review about what is currently known about the hunger hormone ghrelin, its receptor, and its signalling processes. Ghrelin’s well understood role in the modulation of hypothalamic feeding and its recent implications in reward based feeding will also be discussed in detail. Subsequently, the endocannabinoid system’s involvement in the modulation of both hypothalamic and mesolimbic dopamine feeding circuits will be investigated. Lastly, the notion that the interaction between ghrelin and endocannabinoid systems is imperative for controlling feeding within the hypothalamus, which led to our working hypothesis that an interaction between ghrelin and endocannabinoid systems is also essential for modulating food intake within the VTA, will both be presented here and supported by data here within.

List of Abbreviations

Δ^9 -THC, Δ^9 - tetrahydrocannabinol; **2-AG**, 2-arachidonoylglycerol; **α -MSH**, alpha-melanocyte-stimulating hormone; **ACC**, acetyl-coenzymeA carboxylase; **AEA**, anandamide; **AgRP**, agouti-related peptide; **AMG**, amygdala; **AMP**, adenosine monophosphate; **AMPK**, adenosine monophosphate activated protein kinase; **ANOVA**, analysis of variance; **ATP**, adenosine triphosphate; **ARC**, arcuate nucleus; **β -MSH**, beta –melanocyte-stimulating hormone; **BAPTA**, 1,2-bis(o-aminophenoxy) ethane-N,N',N'-tetraacetic acid; **BMI**, body mass index; **BOLD**, blood oxygen level dependent; **CaMKII**, Ca^{2+} / calmodulin-dependent protein kinase II; **cAMP**, cyclic adenosine monophosphate; **CART**, cocaine amphetamine regulated transcript; **cdNA**, complimentary deoxyribonucleic acid **CB**, cannabinoid; **CB-1R**, cannabinoid receptor 1; **CB-1R KO**, CB-1R knockout; **CB-2R**, cannabinoid receptor 2; **CNS**, central nervous system; **CPP**, conditioned place preference; **CPT1 α** , carnitine-palmitoyl-transferase-1 α ; **DEPC**, diethylpyrocarbonate; **DMH**, dorsomedial nucleus; **FAAH**, fatty acid amid hydrolase; **FAS**, fatty acid synthase; **GABA**, gamma-aminobutyric acid; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **GC**, gas chromatography; **GPCR**, G-protein coupled receptors; **GHSR**, growth hormone secretagogue receptor; **GHSR KO**, growth hormone secretagogue receptor knockout; **GOAT**, ghrelin O-acyltransferase; **HIP**, hippocampus; **HYP**, hypothalamus; **IP**, intraperitoneal; **LCFA-CoA**, long chain fatty acid-coenzymeA; **LHA**, lateral hypothalamic area; **MAGL**, monoacylglycerol lipase; **MC₃**, melanocortin-3 receptor; **MC₄**, melanocortin-4 receptor; **mEPSC**, miniature excitatory post synaptic current; **mIPSC**, miniature inhibitory post synaptic current; **mRNA**, messenger ribonucleic acid; **NA**, nucleus accumbens; **NPY**, neuropeptide Y; **NTS**, nucleus of the tractus solitaries; **OX-1**, orexin 1 receptor; **pAMPK**, phosphorylated adenosine monophosphate activated protein kinase; **PFA**, paraformaldehyde; **PFC**, prefrontal cortex; **PKA**, protein kinase A; **PNS**, peripheral nervous system; **POMC**, pro-opiomelanocortin; **PPT**, pedunculo pontine nucleus; **PVN**, paraventricular nucleus; **RT-qPCR**, reverse transcription quantitative polymerase chain reaction; **THL**, tetrahydrolipstatin; **VMN**, ventromedial nucleus; **VP**, ventral pallidum; **VTA**, ventral tegmental area; **WT**, wild-type

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Introduction

Obesity: Weighing in on the Problem

Obesity is a major health epidemic that is considered by many to be the leading preventable cause of death worldwide. Alarming, its prevalence in developed nations has increased substantially, with obesity rates rising almost 50 percent since the 1980's. In 2008, almost 1.5 billion adults (over the age of 20) were categorized as overweight (i.e. body mass index (BMI) equal or greater than 25), with over 500 million of these adults, surpassing obese qualifications (i.e. BMI equal to or greater than 30). The increased frequency of obese and overweight individuals worldwide reflects a concomitant overindulgence in easily available high energy foods, which are often consumed independent of energy needs, as well as a shift to more sedentary lifestyles. While being overweight or obese has many negative consequences, some of the more distressing associated medical problems include increased incidence of type 2 diabetes, cardiovascular disease, atherosclerosis, hypertension, glucose intolerance, dyslipidemia, and cancer. (World Health Organization, 2013)

The negative repercussions of being overweight or obese are not limited to adverse health complications. These metabolic states also convey a significant financial burden. Estimating the financial costs associated with obesity is difficult as it is hard to delimit what constitutes an obesity or overweight related cost; however, studies conducted in 2008 estimate that obesity expenses in Canada range between \$4.6-\$7.1 billion depending on whether the amalgamated medical costs for 8 or 18 chronic diseases most consistently linked to obesity are used (Public Health Agency of Canada, 2011). Studies conducted in the United States, which base their estimates on direct costs (e.g. health services, laboratory test, medication) as well as

indirect costs (e.g. lost work/productivity, insurance) have reported that an estimated 210 billion dollars (i.e. 20.6% of national health expenditures) is spent on obesity related illnesses annually (Cawley & Meyerhoefer, 2012).

The Need for Tight Regulation of Feeding and Energy Balance

The complexity and redundancy in neural circuits responsible for the regulation of feeding behaviours and energy balance makes studying the mechanisms responsible for these processes difficult. However, through the identification of the important brain areas (i.e. nuclei), neurons, peptides, and other chemicals that contribute to these processes a comprehensive understanding of how the central nervous system (CNS) and peripheral nervous system (PNS) collaborate to control these processes is feasible. Historically, environmental changes in an organism's life were highly variable and unpredictable (e.g. food, weather, etc.) making the ability to modulate energy metabolism quickly and alter feeding behaviours so as to promote food procurement safely, essential for survival. To achieve this level of control and to ensure that energy demands to fuel cellular and vital homeostatic processes were satisfied, humans and other higher life forms developed an efficient means of communication between peripheral organs and the CNS. Peripheral hormone signals, which are released from many organs (e.g. fat, liver, pancreas, etc.), provide this necessary communication. Among these, gastrointestinal tract peptides have garnered much research interest due to the robust phenotypic and behavioural changes induced when these peptide systems are enhanced or dampened. There are over 20 different gastrointestinal tract peptides that are known to be involved in feeding responses and energy homeostasis (see Table 1 for examples); however,

ghrelin, will be the predominant hormone discussed in detail in this thesis (please refer to many excellent reviews for detailed descriptions of other peripheral hormones) (Leibowitz & Wortely, 2004; Patton & Mistlberger, 2013; Koleva *et al.*, 2013; Suzuki *et al.*, 2012). In general, gastrointestinal hormones act on organs of the PNS as well as important brain areas responsible for modulating feeding and balancing energy demands and surpluses (Murphy & Bloom, 2006). They are most often released from the gastrointestinal tract or adipose tissue to relay relevant information regarding internal energy states or shortly after food intake to update the brain of incoming nutrients. Once released, these hormones travel through the bloodstream and access their CNS targets via circumventricular organs (i.e. median eminence & area postrema) that are characterized by extensive vasculature and weak blood brain barrier formation or through vagal afferent mechanisms (Sam *et al.*, 2012). The arcuate nucleus (ARC) of the hypothalamus (HYP) and the nucleus of the tractus solitarius (NTS) (projects to HYP) are two well studied regions where peripheral hormones enter the CNS to elicit their intended responses (Sam *et al.*, 2012).

Table 1: Summary of well known gut peptide hormones implicated in feeding

Gut Peptide/ Hormone	Reasons for Secretion	Major Site(s) of Secretion	Main Regions Involved in Mediating Feeding Response	Effect on Food Intake	Other Effects
Amylin	Meal ingestion	Pancreatic β cells	Brain (modulates activity of the serotonin, histamine, & dopaminergic systems)	↓	↓NPY release, ↓ adiposity
Cholecystokinin	Saturated fats, long chain fatty	Small intestine I cells and	Hypothalamus (DMH), brainstem (NTS)	↓	↑PYY release, ↓ ghrelin, ↑ pancreatic

	acids, amino acids	gut L cells	and AP), cortex, vagal afferents, gastric mucosa		secretions and gallbladder bile salts
Ghrelin	Fasted states	X/A-like cells of gastric oxyntic mucosa lining the stomach	Vagal afferents, hypothalamus, brainstem (AP, NTS), VTA	↑	↑ gastric acid and pancreatic secretion, ↑ locomotor activity, ↑ growth hormone secretion
Glucagon	Fasted states, exercise, stress	Pancreatic α cells	Liver, kidney, adipocytes, spleen, pancreas, heart, gut adrenals	↓	↑ stress response, ↑ hepatic glycogenolysis and gluconeogenesis, ↑ energy expenditure
Glucagon-like Peptide-1	Glucose intake	Gut L cells	Hypothalamus, brainstem	↓	↑ insulin release, ↓ blood glucose, delays gastric emptying
Oxyntomodulin	Caloric intake	Gut L cells	Hypothalamus (ARC, PVN, supraoptic nucleus)	↓	↑ increases energy expenditure, ↓ gastric secretion & delays gastric emptying
Pancreatic polypeptide	Caloric intake	Pancreatic islet PP cells	Hypothalamus (ARC, VMH and PVN)	↓	↑ insulin release, ↑ blood glucose, delays gastric emptying,
Peptide tyrosine tyrosine	Caloric intake (especially proteins)	Gut L cells	Hypothalamus (ARC), brainstem	↓	↓ gastric emptying, ↓ of jejunal & colonic motility, ↓ pancreatic secretions

- Note that this is not an inclusive list of all gut peptides
- This table was developed with information reviewed in Suzuki *et al.*, 2012 & Sam *et al.*, 2012

Ghrelin and its Receptor

Of all of the peripheral hormones associated with food related behaviours and energy balance (e.g. appetite, feeding, substrate utilization), ghrelin is one of the most intensely studied. Ghrelin is a 28 amino-acid peptide that is secreted primarily by endocrine gastric X/A-like cells of the oxyntic mucosa lining of the stomach (Kojima *et al.*, 1999; Dornonville de la Cour *et al.*, 2001); however, expression has also been reported in the following organs: ovary (Caminos *et al.*, 2003), placenta (Gualillo *et al.*, 2001), testis (Tanaka *et al.*, 2001), small intestine (Date *et al.*, 2000), pancreas (Volante *et al.*, 2002), kidney (Mori *et al.*, 2000), lymphocytes (Hattori *et al.*, 2001), pituitary (Korbonits *et al.*, 2001), and the brain (Lu *et al.*, 2002; Ferrini *et al.*, 2009). Ghrelin is the endogenous ligand of the seven transmembrane G-protein coupled type 1a growth hormone secretagogue receptor (GHSR) (Howard *et al.*, 1996). This receptor shows constitutive activity and modulates its response to ghrelin by receptor internalization and by altering its transcript expression (Takaya *et al.*, 2001; Camina *et al.*, 2004; Schellekens *et al.*, 2013). Interestingly, ghrelin requires a post-translation transacylation of a medium chain fatty acid (i.e. 6-12C), most commonly an *n*-octanoylation, on the hydroxy group of serine (position 3 of the peptide) for the peptide to be able to bind to GHSR (Bednarek *et al.*, 2000; Gutierrez *et al.*, 2008). The addition of *n*-octanoyl acid changes the conformation of the ghrelin peptide in such a way as to permit efficient binding of ghrelin to the active site (i.e. binding pocket) of the GHSR (Bednarek *et al.*, 2000). Currently, the acylation of ghrelin is known to be mediated solely by a member of the membrane-bound O-acyltransferases called ghrelin O-acyltransferase (GOAT), as evidenced by the complete lack of acylated ghrelin in GOAT knock out (KO) mice (Gutierrez *et al.*, 2008). Furthermore, because GOAT is the only known enzyme

able to activate ghrelin, and that GOAT's expression varies with diet and nourishment, it is suggested that changes in GOAT expression may be one of the molecular switches that provides information about nutrient status (e.g. lipids levels) (Kirchner *et al.*, 2009). Surprisingly, less than 20 % of circulating ghrelin is acylated and thus able to bind to GHSRs to elicit ghrelin's known orexigenic effects (Hosoda *et al.*, 2000). It remains a debate whether the rest, which circulates as des-acyl-ghrelin, is a degradation product or a precursor of the active form or whether it binds to a yet uncharacterised receptor to elicit effects (Hosoda *et al.*, 2000, Ferrini *et al.*, 2009). The GHSR is distributed in various locations within the periphery (i.e. adipose tissue, adrenals, gonads, lung, liver, kidney, pancreas, thyroid, arteries, myocardium, & stomach) and CNS (i.e. HYP, ventral tegmental area (VTA), cerebral cortex, hippocampus (HIP), substantia nigra, raphe nuclei, nodose ganglion, & dorsal vagal complex) (reviewed in, Ferrini *et al.*, 2009). Not surprisingly, the vast distribution of ghrelin and its receptor allow ghrelin to modulate a variety of different processes such as insulin secretion (Broglia *et al.*, 2003), glucose metabolism (Patel *et al.*, 2006; Sun *et al.*, 2007), anxiety (Asakawa *et al.*, 2001; Schellekens *et al.*, 2012), and reward (Abizaid *et al.*, 2006, Schellekens *et al.*, 2012), in addition to its known role in modulating feeding behaviour (Tschop *et al.*, 2000). Ghrelin is known to stimulate both food intake and body weight gain upon systemic and central administration (Tschop *et al.*, 2000; Wren *et al.*, 2001a; Wren *et al.*, 2001b). Although ghrelin has the capacity to enhance body weight and adiposity in an attempt to manage long term energy balance, a process that in part relies on decreasing lipid oxidation in the periphery, the present thesis focuses primarily on ghrelin's effects on feeding (Tschop *et al.*, 2000; Theander-Carrillo *et al.*, 2006).

GHSR Signaling

Understanding the intracellular signaling pathways that are initiated or affected upon ghrelin binding to its receptor (i.e. GHSR), which elicit changes in feeding behavior and energy homeostasis, is of paramount importance, especially when attempting to comprehend the major players, pathways, and corresponding interactions (i.e. indirect or direct) that contribute to these physiological changes. Several signaling pathways have been shown to be activated upon ghrelin binding to its receptor; however, it is important to note that many of the studies that have contributed to the elucidation of these pathways have used a variety of unique cell types and/or tissues. These pathways include the phospholipase C signaling pathway (elucidated in rat pituitary cells) (Chen, 2000), the protein kinase A (PKA) pathway (discovered in neuropeptide Y (NPY) producing neurons of the arcuate nucleus) (Kohno *et al.*, 2003), and the cyclic adenosine monophosphate (cAMP) element binding protein (CREB) pathway (revealed in transfected COS-7 & human embryonic kidney 293 cells) (Holst *et al.*, 2003); although, one of the best understood is the adenosine mono-phosphate activated protein kinase (AMPK) pathway (Andersson *et al.*, 2004; Kola *et al.*, 2005). This pathway has gained considerable support following several western blot and AMPK assay studies which methodically identified important enzymes involved in mediating ghrelin's orexigenic response within dissected hypothalami (refer to Figure 1) (Andersson *et al.*, 2004; Kola *et al.*, 2005; Kola *et al.*, 2008). Interestingly, within the HYP, neurons use the quantity of cytoplasmic long chain fatty acid-coenzymeA (LCFA-CoA) molecules as an indicator of energy stores (Hardie & Carling, 1997; Andersson *et al.*, 2004; Kola *et al.*, 2005; Lopez *et al.*, 2008). An accumulation of these molecules within the cytoplasm represents a positive energy balance while low levels signify a

negative energy balance (i.e. high AMP/ATP ratio) (Hardie & Carling, 1997; Andersson *et al.*, 2004). These LCFAs are quickly converted to LCFA-CoAs following entry into the cell by an enzyme called fatty acid synthase (FAS) before they are subsequently shuttled into the mitochondria by carnitine-palmitoyl-transferase-1 α (CPT1 α) to aid in the generation of ATP through β -oxidation processes (Obici *et al.*, 2002; Obici *et al.*, 2003). The CPT1 α mitochondrial shuttling enzyme is allosterically inhibited by malonyl-Co-A, which is produced when a carboxyl group is transferred onto acetyl-CoA via acetyl-coenzymeA carboxylase (ACC) (Ha *et al.*, 1998; McGarry *et al.*, 1978). According to several reports, when ghrelin binds to its endogenous receptor within hypothalamic regions, AMPK is activated (Andersson *et al.*, 2004; Kola *et al.*, 2005; Kola *et al.*, 2008; Lim *et al.*, 2013). Although the exact mechanism in which ghrelin activates AMPK within the HYP is unknown, it is speculated that Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) may be activated by increased intracellular Ca^{2+} that accompanies GHSR activation (Kola, 2008). Once activated, CaMKII is thought to phosphorylate AMPK at its corresponding threonine activation site (Kola, 2008). Following AMPK phosphorylation, pAMPK (phosphorylated AMPK) initiates 2 separate processes that lower cytoplasmic LCFA-CoA, which ultimately result in the stimulation of food intake (Andersson *et al.*, 2004; Kola *et al.*, 2005; Kola *et al.*, 2008; Lim *et al.*, 2013). First, activated AMPK (pAMPK) phosphorylates and inhibits ACC, mitigating the conversion of acetyl-CoA to malonyl-CoA, effectively disinhibiting CPT1 α (Kola *et al.*, 2005; Andersson *et al.*, 2004). In response, this enzyme shuttles cytoplasmic LCFA-CoA molecules into the mitochondria for β -oxidation thus reducing the cytoplasmic levels of LCFA-CoA (Obici *et al.*, 2003). This reduction in cytoplasmic levels of LCFA-CoA serves as a starved energy depleted signal consequently leading to the stimulation of food intake to replenish

energy stores (Obici *et al.*, 2002; Obici *et al.*, 2003). Second, activated pAMPK also inhibits FAS, thereby preventing the transfer of CoA from malonyl-CoA to LCFA (Lopez *et al.*, 2008). This further contributes to the promotion of food intake by preventing the replenishment of cytoplasmic LCFA-CoA levels (Lopez *et al.*, 2008). In contrast, it is important to note that although it is highly supported that ghrelin's orexigenic effects are in part mediated by the activation of AMPK signaling cascades within the hypothalamus; this is not always the case. Increases in food intake caused by high ghrelin levels, enhanced endogenously (by fasting) and by external administration, have been observed in mice without increases in AMPK activity or phosphorylation of its downstream targets (Verhulst *et al.*, 2012).

Figure 1: Hypothetical interactions of ghrelin, leptin, and cannabinoids on hypothalamic AMPK activity. Note that both GHSR-1a and CB-1 receptor activation, possibly by heterodimerization, leads to activation of AMPK and the overall stimulation of food intake; whereas, leptin inhibits AMPK activity to reduce food intake.

In contrast, ghrelin is known to inhibit AMPK activity in peripheral tissues such as the liver, skeletal muscles and adipose tissue, leading to decreased glucose uptake and fatty acid oxidation and the promotion of fatty acid synthesis (Kola *et al.*, 2005; Hardie & Sakamoto, 2006). This is consistent with the observation that ghrelin promotes adiposity by promoting carbohydrate utilization over fat (Tshop *et al.*, 2000). Ultimately, it seems as if the AMPK pathway is intimately involved in mediating energy homeostasis and feeding behaviors since fluctuations in AMPK activity and associated downstream enzymes reflect changes in energy status and are influenced by peripheral hormone levels such as ghrelin (Andersson *et al.*, 2004, Minokoshi *et al.*, 2002; Minokoshi *et al.*, 2004).

Hypothalamic Control of Feeding

The HYP, which consists of distinct nuclei such as the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial nucleus (DMH), ventromedial nucleus (VMN), and the ARC, is the most extensively studied and important brain region involved in coordinating the regulation of homeostatic food intake and maintenance of energy homeostasis. It integrates information, collected both centrally and from the periphery (e.g. peripheral hormones), regarding energy status and stores and initiates appropriate mechanisms and behaviours to restore homeostasis (i.e. feeding or substrate utilization) (reviewed in, Abizaid & Horvath, 2008). This thesis' primary focus is to elucidate what drives non-hypothalamic feeding with an overall emphasis on how ghrelin and endocannabinoids interact to modulate feeding behaviours within the mesolimbic dopamine circuit. Although all regions of the HYP contribute to the regulation of homeostatic feeding and energy balance, as exemplified by the extreme

changes in phenotype (PVN, VHM, DMH- hyperphagic & obese; LHA- hypophagic & lean) caused by lesion studies which target other hypothalamic nuclei, the contributions of ghrelin to promoting food intake in the ARC is only briefly outlined below (Brobeck *et al.*, 1943; Hetherington & Ranson, 1940; Hetherington, 1944; Anand & Brobeck, 1951). Details concerning the identity and location of cell types, neuropeptides, innervations, projections, and behavioural studies associated with the aforementioned hypothalamic nuclei can be found in some excellent recent reviews (Abizaid & Horvath, 2008; Leibowitz & Wortely, 2004; Gao & Horvath, 2007; Lenard & Berthoud, 2008; Sanchez-Lasheras *et al.*, 2010).

Within the ARC, two main opposing cell types modulate feeding behaviours in response to endogenous signalling, neuropeptide Y/agouti-related peptide/gamma-aminobutyric acid (NPY/AgRP/GABA) neurons, which promote appetite when activated, and pro-opiomelanocortin/cocaine amphetamine regulated transcript (POMC/CART) neurons, which encourage satiety when activated (Boston *et al.*, 1997; Hahn *et al.*, 1998; Ellacott & Cone, 2004). Ghrelin's orexigenic ability is driven in part by its capacity to increase the activity of NPY/AgRP/GABA neurons directly (Cowley *et al.*, 2003) as well as indirectly by increasing the number of excitatory synapses that contact these neurons (Horvath, 2006). In contrast, ghrelin has the opposite effects on POMC neurons, encouraging decreased excitatory inputs onto them in addition to promoting the activity of GABA inhibitory synapses onto these satiety promoting neurons (Cowley *et al.*, 2003; Horvath, 2006; Dietrich & Horvath, 2013). During times of energy deficiency (i.e. fasted state) circulating ghrelin levels are elevated leading to the activation of NPY/AgRP/GABA neurons and the suppression of POMC/CART neurons (Abizaid & Horvath, 2008). Once activated the NPY/AgRP/GABA neurons stimulate feeding through

multiple mechanisms. First, NPY/AgRP/GABA neurons transcribe and release AgRP, which is known to antagonize the binding of alpha-melanocyte-stimulating hormone (α -MSH) and β -MSH (produced by POMC/CART neurons) at melanocortin receptors (MC_3 & MC_4) located in the PVN, LH, and DMH (dampens anorectic outputs from these regions) (Adan *et al.*, 1994; Mountjoy *et al.*, 1994; Sahm *et al.*, 1994; Ollmann *et al.*, 1997; Biebermann *et al.*, 2006; Aponte *et al.*, 2011). Likewise, NPY/AgRP/GABA neuron activation results in the release of orexigenic NPY from projections that exist in multiple hypothalamic nuclei such as the PVN, VMH, and LH (Stanley & Leibowitz, 1984; Mountjoy *et al.*, 1994; Sahm *et al.*, 1994; Baskin *et al.*, 1999; Goa & Horvath 2007; Abizaid & Horvath, 2008). The release of NPY initiates behaviours favouring food procurement and feeding (Abizaid & Horvath, 2008). Lastly, NPY/AgRP/GABA neurons project unidirectional GABA inhibitory synapses onto neighbouring POMC/CART neurons within the ARC to reduce the activity of these neurons and effectively promote feeding responses (Cowley *et al.*, 2001, Tong *et al.*, 2008). These effects are reversed during a meal as nutrients and hormone satiety signals rise (Dietrich & Horvath, 2013). Eventually this leads to POMC/CART neurons becoming less inhibited by GABA inhibitory synapses of NPY/AgRP/GABA neurons and their secreted anorectic peptides (i.e. α -MSH and β -MSH) are less inhibited by AgRP at melanocortin receptors in target regions (e.g. PVN, LH, DMH, etc.) (Abizaid & Horvath, 2008). Ultimately, increased production of α -MSH, β -MSH, and CART from POMC neurons and decreased NPY/AgRP/GABAergic tone results in an overall suppression of orexigenic pathways and the promotion of feelings of satiety (Abizaid & Horvath, 2008).

Ghrelin and Reward:

The ability of ghrelin to stimulate food intake is not solely driven by its action within the aforementioned hypothalamic nuclei. Recently, it has been demonstrated that ghrelin plays an important role in the hedonic aspect of feeding behaviours and contributes to the induction of motivational behaviours that stimulate food intake, especially that of palatable foods (reviewed in Abizaid, 2009; Perello & Zigman, 2012; van Zessen *et al.*, 2012). These studies, in addition to the location and high levels of GHSR-1a transcript highlighted throughout the mesolimbic dopamine system, which consist of dopaminergic neurons in the VTA and their corresponding neural projections to cortico-limbic structures such as the nucleus accumbens (NA), PFC, HIP, and the amygdala (AMG), have identified the mesolimbic dopamine system as a prime candidate for mediating ghrelin's extra-hypothalamic effects (see Figure 2) (Guan *et al.*, 1997; Saper *et al.*, 2002; Zigman *et al.*, 2006; Field *et al.*, 2007).

Figure 2: Over-simplified depiction of the mesolimbic dopamine system in a rat brain. Note that many connections are omitted for simplicity. AMG, amygdala; HIP, hippocampus; LH, lateral hypothalamus; mPFC, medial prefrontal cortex; NA, nucleus accumbens; PPT, pedunculopontine nucleus; VP, ventral pallidum; VTA, ventral tegmental area.

Interestingly, ghrelin seems to modify food preference in rodents, as rats will choose a high-fat diet over a carbohydrate rich diet following intracerebroventricular injection of ghrelin; while, peripheral ghrelin causes a preferred selection of sweet sugar solution in wild-type (WT) but not GHSR KO mice (Shimbara *et al.*, 2004; Disse *et al.*, 2010). Furthermore, disruption of the ghrelinergic system (using GHSR-KO mice or ghrelin receptor antagonists) causes a significant decrease in the consumption of palatable foods but not necessarily regular chow compared to controls (Egecioglu *et al.*, 2010; Landgren *et al.*, 2011).

Moreover, the involvement of the ghrelin system in reward based feeding behavior is exemplified in conditioned place preference (CPP) and operant conditioning tests that use food rewards. The development of CPP for rewarding foods (high fat diet or chocolate) over less rewarding conditions (i.e. standard chow or no reward) has been observed with both the administration of ghrelin and stress induced endogenous increases of ghrelin (Perello *et al.*, 2010; Chuang *et al.*, 2011). Conversely, studies run using GHSR antagonists or GHSR KO rodents demonstrate the inability of rodents to acquire CPP for rewarding foods (Perello *et al.*, 2010; Chuang *et al.*, 2011). Additionally, rodents treated with peripheral ghrelin increase the number of times they will bar press for palatable rewards over vehicle treated animals, whereas, administration of a GHSR antagonist has the opposite effect (Perello *et al.*, 2010; Finger *et al.*, 2012).

Research focused on elucidating the neural circuits mediating ghrelin's influence on reward based feeding behaviours has primarily focused on the mesolimbic dopamine system, particularly the VTA. This is primarily due to the high expression of GHSR within the VTA and the fact that dopamine neurons that originate within this region are known to project to and

modulate the activity of numerous cortico-limbic areas (refer to Figure 2) (Saper *et al.*, 2002; Abizaid *et al.*, 2006). Abizaid and colleagues demonstrated that ghrelin was able to activate dopaminergic neurons within the VTA by binding to GHSR located on these neurons and by enhancing the ratio of excitatory versus inhibitory synapses contacting these neurons (Abizaid *et al.*, 2006). Consequently, this stimulated an increase in dopamine turnover within the NA (Abizaid *et al.*, 2006). Moreover, they discovered that ghrelin increased the activity of these dopamine cells within WT but not GHSR KO mice (Abizaid *et al.*, 2006). They complimented their molecular and electrophysiological data with behavioural studies showing that intra-VTA ghrelin infusions increased feeding (Abizaid *et al.*, 2006). Furthermore, they showed that intra-VTA infusion of a GHSR antagonist suppressed the appetite inducing ability of peripherally administered ghrelin as well as endogenously high ghrelin relative to controls (Abizaid *et al.*, 2006).

Following the above study a series of behavioural experiments were conducted to examine the feeding effects of ghrelin within the VTA. King and colleagues found that chronic intra-VTA administration of ghrelin stimulates a sustained enhancement in food intake that increases dose dependently (King *et al.*, 2012). Interestingly, they found that intra-VTA infusions of a GHSR antagonist selectively decreased the intake of a high fat diet but not the intake of protein or carbohydrate rich diets (i.e. when all diets were freely available) (King *et al.*, 2012). Additionally, they reported that chronic administration of ghrelin within the VTA significantly increased while chronic infusions of GHSR antagonist reduced the amount of work non-fasted rats were willing to expend to earn a chocolate pellet (fixed-ratio paradigm) (King *et al.*, 2012). Consistent with this, animals with VTA lesions eat less palatable food but not regular

chow when ghrelin is administered peripherally (Egecioglu *et al.*, 2010). Furthermore, these lesioned animals are less motivated to search for food when ghrelin is centrally administered than corresponding controls (Egecioglu *et al.*, 2010). When a progressive ratio paradigm is used, where rats are required to press a lever more times with each given reward, intra-VTA ghrelin is shown to increase the breakpoint (number of presses within certain time frame); whereas, this effect is attenuated following VTA dopamine neuron specific lesions (Weinburg *et al.*, 2011). Similarly, peripheral ghrelin injections in Cre-Lox transgenic mice, which only express normal levels of GHSR in dopamine neurons (i.e. TH-containing neurons), such as those found in the VTA, partially rescues the increase in food intake and development of CPP for a high fat diet that is seen when ghrelin is peripherally administered in WT mice (i.e. significantly higher than that seen in GHSR KO mice) (Chuang *et al.*, 2011).

Interestingly, it has also been demonstrated that intravenously administered ghrelin activates these regions in humans, as evidenced by increased neuronal responses from mesolimbic areas, detected using functional magnetic resonance imaging, when volunteers are shown pictures of food (Malik *et al.*, 2008).

Overall, these experiments lend evidence to the important role that the ghrelin system plays in hedonic feeding within the mesolimbic dopamine system.

The Discovery of the Endocannabinoid System

Cannabis sativa has been used medicinally for thousands of years to treat rheumatic and menstrual pain; however, its appetite inducing effects were only discovered a couple of centuries ago (Di Marzo, 2006). Interestingly, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the chemical

responsible for a majority of the psychoactive properties of marijuana, was discovered to be the component of *Cannabis sativa* that causes appetite stimulation (Gaoni & Mechoulam, 1964). The first endogenous receptor for Δ^9 -THC (i.e. cannabinoid receptor 1) was discovered and cloned almost 30 years after Δ^9 -THC's chemical characterization using Δ^9 -THC labelled analogues (i.e. HU-210) and screening banks of orphan G-protein-coupled receptors (GPCRs) (Devane *et al.*, 1988; Matsuda *et al.*, 1990). Shortly after a second seven transmembrane G protein-coupled receptor was cloned (i.e. CB-2 receptor) that also bound to Δ^9 -THC and other endogenous compounds (Munro *et al.*, 1993; Di Marzo *et al.*, 1995). These endogenous compounds, which share an affinity for the CB receptors, are collectively termed endocannabinoids and are all derivatives (i.e. amides, ethers, & esters) of long chain polyunsaturated fatty acids (Di Marzo *et al.*, 2004). It is important to note that CB-1Rs, which are found predominantly in the CNS, are the most abundant known GPCR in the brain; whereas, CB-2Rs, are more commonly found in the periphery (e.g. spleen, thymus, tonsils, pancreas, etc.) and on immune cells (Herkenham *et al.*, 1991; Howlett, 2002). Consistent with the widespread expression of CB receptors, the endocannabinoid system is associated with various physiological processes such as neural development, immune functioning, synaptic plasticity, learning, pain, emotional states, motivation, and reward (reviewed in Viveros *et al.*, 2008). However, its role in feeding will be the focus in the present thesis following a brief introduction of the important aspects of the endocannabinoid system.

Endocannabinoids – The Retrograde Signalers

The arachidonic acid derivatives anandamide (*N*-arachidonylethanolamine) (AEA) and 2-arachidonoylglycerol (2-AG) were the first two endocannabinoids discovered and remain the two most widely studied (Devane *et al.*, 1992; Sugiura *et al.*, 1995). Within the CNS, these two endocannabinoids are reported to be synthesized on demand from lipids of the post-synaptic neuronal membrane in response to intracellular increases in Ca^{2+} (Di Marzo *et al.*, 2004). Concomitant increases in cyclic AMP and intracellular Ca^{2+} that occur as a result of neuronal activation stimulate the enzymes necessary for the “de novo” synthesis of AEA and 2-AG (Di Marzo *et al.*, 2004). Generally, these endocannabinoids are thought to diffuse across the synaptic cleft from the activated post-synaptic neuron where they are synthesized to the pre-synaptic neuron where they bind membrane bound CB-1Rs to suppress neurotransmitter release (Alger, 2002). This model of cannabinoid action is supported by substantial electrophysiological and biochemical studies that were conducted with both acute brain slices and cultured neurons. First, CB-1Rs are predominantly localized to pre-synaptic terminals; whereas, the enzymes that are required for the formation of 2-AG and AEA are located primarily in post-synaptic neurons (Kano *et al.*, 2009). Second, following post-synaptic neuron stimulation, neurotransmitter release from pre-synaptic cells is reduced when cannabinoid agonists (e.g. WIN55, 212-2) bath the slices; whereas, pre-treatment with synthetic cannabinoid antagonists (e.g. SR141716A) prevent inhibition of neurotransmitter release from pre-synaptic terminals (Ohno-Shosaku *et al.*, 2001). Third, Wilson and Nicole demonstrated that liberating calcium stores within a post-synaptic cell *via* photolysis is sufficient to induce endocannabinoid signalling and prevent neurotransmitter release from pre-synaptic neurons; whereas, inhibiting

calcium release blocks this inhibition (Wilson & Nicole, 2001). Lastly, the lipophilic nature of endocannabinoids and the inability of botulinum toxin to block endocannabinoid inhibition of neurotransmitter release support a role for a vesicle independent diffusion of endocannabinoids across the synaptic cleft to elicit their effects (Wilson & Nicole, 2001).

Mechanisms exist to clear (transport) and recycle (degrade) left over endocannabinoids from the extracellular space following their retrograde release and stimulation of pre-synaptic CB-1Rs. Although this process is not extremely well understood, it is thought AEA does one of three things to get into post-synaptic neurons where the degradative enzyme that can break it down mostly reside (Di Marzo *et al.*, 2004). Research suggests that AEA's transport across the cell membrane is either mediated via a carrier protein (Fegley *et al.*, 2004), through endocytotic mechanisms (McFarland *et al.*, 2004) or by passive diffusion (Glaser *et al.*, 2003). Less is known about how 2-AG is cleared from the extracellular space but some hypothesize that it may share the same system or mechanism that AEA uses (Beltramo & Piomelli, 2000; Beltramo *et al.*, 1997). In contrast, there is also evidence supporting that 2-AG must have its own transporter or passively diffuse across the neuronal membrane as monoacylglycerol (MAGL), the enzyme that is mostly responsible for its degradation, is found predominantly in the pre-synaptic neuron (Gulyas *et al.*, 2004). Proceeding entry into the cell these endocannabinoids are broken down by both hydrolysis and oxidation processes (Vandevorde & Lambert, 2007). AEA is hydrolyzed by an enzyme called fatty acid amid hydrolase (FAAH), whereas, 2-AG is hydrolyzed by MAGL (Vandevorde & Lambert, 2007). Furthermore, cyclooxygenase and lipoxygenase enzymes facilitate the oxidization of 2-AG and AEA at their corresponding arachidonic acid groups (Vandevorde & Lambert, 2007).

Suppression of Transmitter Release and CB-1R Signalling

Activation of CB-1Rs has been reported to cause a multitude of intracellular changes that contribute to the known neurotransmitter release inhibition seen in glutamate, GABA, dopamine, serotonin, acetylcholine, norepinephrine, glycine, and cholecystokinin neurons (reviewed in, Kano *et al.*, 2009). Most of these processes rely on CB-1R associated $G_{i/o}$ family of G proteins as supported by studies that show disinhibition of neurotransmitter release when CB-1Rs are activated by CB-1R agonists in the presence of pertussis toxin (i.e. inactivates G_i and G_o G- proteins) (Pertwee, 1997). However, its interaction with G_s has also been observed in pertussis toxin treated conditions (Pertwee, 1997). Traditionally, one of the most frequently reported intracellular events initiated upon CB-1R activation is the inhibition of adenylyl cyclase (Demuth & Molleman, 2006). This inhibition leads to a corresponding drop in cAMP levels and a consequential decrease in PKA activity; however, recent studies show that CB-1R activation can lead to the inhibition or activation of adenylyl cyclase depending on which of the 6 isoforms is present in the tissue or cell culture studied (Demuth & Molleman, 2006). In general, CB-1R stimulation leads to the activation of A-type and inwardly rectifying potassium channels while inhibiting N-, L-, and P/Q type calcium channels (Kano *et al.*, 2009). Interestingly, there is also evidence that more direct effects on vesicle release machinery may contribute to the inhibition of neurotransmitter release as tetrodotoxin (i.e. blocks voltage gated Na channels) studies have reported lower frequencies of miniature excitatory post synaptic currents (mEPSC) and miniature inhibitory post synaptic currents (mIPSC) when CB-1 agonists are applied to neuronal slices (Schlucker & Kathmann, 2001). In addition, CB-1R activation leads to the initiation of many pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-

kinase (PI3K) signalling cascades (Kano *et al.*, 2009). Moreover, CB-1R activation is known to increase AMPK activity in the HYP but decrease its activity in fat tissue and liver as seen with GHSR activation (see Figure 1) (Lim *et al.*, 2013). There have been a vast amount of studies that have been conducted on diverse tissues and cultures in an attempt to identify what pathway is responsible for inhibiting neurotransmitter release; however, it seems that a combination of pathways contribute to endocannabinoid inhibition of neurotransmitter release and that this inhibition may be achieved through different mechanisms depending on the cell types and conditions used (Demuth & Molleman, 2006).

Endocannabinoids Stimulate Food Intake

Although significant anecdotal evidence on the appetite inducing effects of marijuana had accumulated by the late 1900's, it was the discovery that Δ^9 -THC and other endogenously found compounds bound to the same CB-1Rs to induce these hyperphagic behaviours that led to an explosion in empirical cannabinoid research (Gaoni & Mechoulam, 1964; Devane *et al.*, 1988; Matsuda *et al.*, 1990; Munro *et al.*, 1993; Di Marzo *et al.*, 1995). Interestingly, synthetic compounds that bind to CB-1Rs are still taken advantage of in modern medicine to attenuate feelings of nausea, stimulate food intake, and mitigate weight loss in cancer and AIDS patients (Di Marzo, & Matias, 2005). A plethora of reputable biochemical, genetic, and behavioural studies investigating feeding behaviours lend support to the important role that the endocannabinoid system plays in modulating feeding. First, the peripheral administration of Δ^9 -THC, 2-AG, and AEA are known to stimulate food intake and enhance feeding behaviours in rodents (Di Marzo *et al.*, 1998; Williams & Kirkham, 1999; Williams & Kirkham, 2002a; Kirkham

et al., 2002; Hao *et al.*, 2000). Second, administration of endocannabinoids or synthetic agonists of the CB-1R within important feeding centers (e.g. HYP, NA) similarly encourages an increase in food intake (Jamshidi & Taylor, 2001; Kirkham *et al.*, 2002; Miller *et al.*, 2004). In contrast, pre-treatment of rodents with a CB-1R antagonist (e.g. rimonabant) blocks both the systemic and central appetite stimulating effects of CB-1R agonists thus confirming that the increases in food intake observed are mediated specifically through CB-1Rs (Rinaldi-Carmona *et al.*, 1994; Williams & Kirkham, 1999; Williams & Kirkham, 2002b). Moreover, peripheral injections of rimonabant can also independently cause a marked reduction in sucrose intake in rats (Arnone *et al.*, 1997). Third, mice lacking functional CB-1Rs are known to have reduced re-feeding following a 24 hour fast compared to controls (Di Marzo *et al.*, 2001). Likewise, CB-1R deficient mice do not decrease their food intake when rimonabant is administered intraperitoneally; whereas, controls demonstrate significantly decreased food intake (Di Marzo *et al.*, 2001).

Endocannabinoids Important in Food Reward

Early studies report that CB-1R antagonists preferentially reduce the intake of palatable foods over regular chow in WT rodents, while cannabinoid receptor deficient mice also demonstrate decreased consumption of palatable foods relative to controls (Arnone *et al.*, 1997; Simiand *et al.*, 1998; Poncelet *et al.*, 2003). In rats, it has been shown that central administration of CB-1R agonists (2-AG, AEA, Δ^9 -THC) increases the number of licks for a 10% sucrose solution; whereas, rimonabant decreases the number of licks compared to controls (i.e. water) (Higgs *et al.*, 2003). A more in depth analysis of the licking microstructure suggests that the licks of the CB-1R agonist treated animals' mimic licks observed when more palatable

solutions are consumed; whereas, those treated with rimonabant licked as if the solution was less palatable (Higgs *et al.*, 2003). Studies looking at hedonic responses, scored by monitoring innate ingestive responses to aversive (quinine) and rewarding foods (sucrose pellets) found that CB-1R agonists decreased the rejection of aversive solutions and increased positive ingestive liking responses of rewarding foods in rats (Jarret *et al.*, 2005; Jarret *et al.*, 2007). This effect was attenuated in rimonabant treated animals (Jarret *et al.*, 2005; Jarret *et al.*, 2007). Furthermore, when CB-1R antagonists were administered alone they diminished hedonic reactions to sucrose and increased dislike of quinine (Jarret *et al.*, 2005; Jarret *et al.*, 2007). Together these experiments support a role for the endocannabinoid system in contributing to the hedonic aspects of food reward.

Many studies have used operant conditioning paradigms to investigate the role that endocannabinoids play in the motivational aspect of feeding behaviour. Two of the most common are the progressive ratio paradigm, in which rats are required to bar press a greater number of times following each reward to get the subsequent treat, and the fixed ratio paradigm, in which rats only receive a reward every predetermined number of lever presses. In the former paradigm it is found that CB-1R agonists dose dependently increase the amount of work rats are willing to expend to receive food rewards while CB-1R antagonists demonstrate a dose dependent decrease (Solinas & Goldberg, 2005). This effect is also mimicked in mice as CB-1R knockout (CB-1R KO) mice decrease their break-point (i.e. point at which animals stop responding) relative to WT mice (Sanchis-Segura *et al.*, 2004). Furthermore, rimonabant treated animals demonstrate a reduced fixed ratio operant responding efficiency when responding for palatable and standard chow rewards (Perio *et al.*, 2001). Interestingly, it has been shown that

administration of CB-1R agonists can induce feeding when animals are not motivated to eat (i.e. completely satiated) (Kirkham, 2009). Consequently, these agonists are reliably observed to reduce the duration of time between meals (Kirkham, 2009). Similarly, humans report higher subjective feelings of hunger with endocannabinoid system stimulation and significantly reduced feelings of hunger and desire to eat following rimonabant treatment (Kirkham, 2009). These experiments support a role for endocannabinoids in enhancing the incentive salience of food.

In agreement with the involvement of endocannabinoids in food motivation and reward, CB-1Rs, CBs, and degradative enzymes are expressed in brain regions traditionally associated with mediating these processes such as those belonging to the mesolimbic dopamine system (see Figure 2) (NA, VTA, HIP, etc.) (Herkenham *et al.*, 1991; Matsuda *et al.*, 1993; Romero *et al.*, 2002; Kirkham *et al.*, 2002). In support of this, CB-1Rs show similar distribution to D1 and D2 receptors within the mesolimbic dopamine system (Hermann *et al.*, 2002). Interestingly, studies demonstrate that intravenous administration of CB-1R agonists cause a robust hyperphagic effect and an increase in dopamine levels (i.e. similar to those observed with drugs of abuse) within the shell of the NA of freely moving rats (Solinas *et al.*, 2006; Kirkham *et al.*, 2002). Furthermore, natural enhancement of dopamine levels within the NA that accompany the presentation of palatable foods is completely attenuated by the administration of rimonabant (Melis *et al.*, 2007).

Although ghrelin's ability to stimulate feeding once targeted to the VTA is fairly well recognized, the role of the endocannabinoid system in modulating VTA driven feeding effects remains poorly understood. Interestingly, the presence of CB-1Rs within the VTA, behavioural &

pharmacological manipulations, and accumulating electrophysiological data advocate that the endocannabinoid system is likewise intimately involved in controlling the activation of VTA dopamine neurons (Szabo *et al.*, 2002; Melis *et al.*, 2004; Oleson *et al.*, 2012; Labouèbe *et al.*, 2013). In support of this, intra-tegmental rimonabant (i.e. 200 ng) decreases while JZL 184, a drug which inhibits MAGL (i.e. 2-AG degradative enzyme) to increase 2-AG levels, increases dopamine neurotransmission in the NA core (Oleson *et al.*, 2012). Moreover, intra-VTA rimonabant treatments consequently reduce whereas intra-VTA JZL 184 treatments increase reward seeking behaviours in intra-cranial self-stimulation and food self-administration tasks (Oleson *et al.*, 2012). Furthermore, intravenous administrations of CB-1 agonists enhance VTA dopamine neuron activity (French *et al.*, 1997; Gessa *et al.*, 1998).

Two different studies support a role for endocannabinoids in modifying pre-synaptic release onto VTA dopamine neurons. Szabo and colleagues highlighted that CB-1R agonists caused inhibition of IPSC in VTA neurons (>75% are dopaminergic), an effect reversed when rimonabant was added to the bath (Szabo *et al.*, 2002). Conversely, Melis and colleagues demonstrated the ability of CB-1R agonists to decrease EPSC onto VTA dopamine neurons by preventing glutamate release onto these neurons, an effect that was also abolished by pre-treatment with rimonabant (Melis *et al.*, 2004). These electrophysiology experiments have confirmed that endocannabinoids are released from VTA dopamine neurons, in response to enhanced intracellular Ca^{2+} (within these VTA dopamine neurons), and travel in a retrograde manner where they bind to CB-1Rs located on both glutamatergic and GABAergic VTA inputs (Wilson & Nicoll, 2001; Szabo *et al.*, 2002; Melis *et al.*, 2004; Lupica & Riegel, 2005). Once bound, they mitigate neurotransmitter release from these neurons, resulting in an overall

activation or suppression of VTA dopamine neurons depending on the ratio of excitatory to inhibitory synapses contacting them (Szabo *et al.*, 2002; Melis *et al.*, 2004; Labouèbe *et al.*, 2013).

Taken together, these studies demonstrate that endocannabinoids may act at many locations within the mesolimbic dopamine system to modulate food reward; however, it is clear that modulation of the endocannabinoid system within the VTA significantly alters the activity of VTA dopamine neurons and hedonic feeding through the manipulation of dopamine release in target regions (e.g. NA) (Oleson *et al.*, 2012). More specifically they suggest that blockage of CB-1Rs within the VTA decreases the activation of VTA dopamine neurons and hence dampens dopamine turnover in target areas to reduce motivation for rewards.

Interaction of Ghrelinergic and Cannabinoid Systems in the Hypothalamus

Endocannabinoid levels in the rat limbic forebrain and HYP are highly correlated with feeding status. Endocannabinoid levels peak in the fasted state and decline shortly after feeding as satiation is approached (Kirkham *et al.*, 2002). Moreover, endocannabinoid levels steadily rise as the duration between meals increases (Kirkham *et al.*, 2002). The fact that endocannabinoid levels mimics ghrelin's expression levels, and that cannabinoid and ghrelin receptors are found in many important brain regions associated with the regulation of food intake and motivated behaviours (e.g. HYP, VTA), suggests that these two systems interact to modulate feeding (Herkenham *et al.*, 1991; Guan *et al.*, 1997). Tucci and colleagues provided the first substantial evidence that this relationship existed when they demonstrated that pre-treatment with a sub-anorectic dose of rimonabant could completely attenuate the increase in

food intake that is observed with intra-PVN injections of ghrelin (Tucci *et al.*, 2004). It was proposed that one of the mechanisms by which ghrelin and endocannabinoids stimulate food intake was by activating AMPK signalling cascades (Kola *et al.*, 2005). This was hypothesized as both ghrelin and cannabinoids (2-AG, AEA, & Δ^9 -THC) administered separately enhance AMPK activity and the phosphorylation of its downstream targets (i.e. ACC) (Kola *et al.*, 2005). Furthermore, it was demonstrated that in the absence of functional CB-1Rs, using CB-1R deficient mice, that ghrelin is unable to induce its characteristic appetite stimulating effects following its central administration (Kola *et al.*, 2008). Although intraperitoneal (IP) ghrelin was able to significantly elevate hypothalamic AMPK activity in WT animals, AMPK levels in both rimonabant treated and CB-1R KO mice were similar to controls (Kola *et al.*, 2008). Interestingly, peripheral administration of ghrelin increases 2-AG levels in HYP (i.e. whole hypothalami) of WT but not CB-1R KO mice (Kola *et al.*, 2008). Likewise, peripheral co-administration of rimonabant and ghrelin is sufficient to mitigate ghrelin's ability to increase 2-AG content within the HYP (Kola *et al.*, 2008). Using electrophysiological experiments it was discovered that ghrelin treatment inhibits mEPSC onto PVN parvocellular neurons (i.e. whole cell patch recordings), which when activated are known to have an inhibitory effect on feeding (Kola *et al.*, 2008). Intriguingly, this inhibition is lost by administration of compounds known to inhibit endocannabinoid signalling such as rimonabant, BAPTA (calcium chelator), and tetrahydrolipstatin (inhibits 2-AG synthesis) (Gao & Horvath, 2007; Kola *et al.*, 2008). The above experiments advocate that within the HYP ghrelin's ability to increase 2-AG content, AMPK activation and food intake is reliant on a functional cannabinoid system. Conversely, Lim and colleagues demonstrated that both ghrelin and CB-1R agonist (HU210) enhance hypothalamic

AMPK activity in WT mice but that this activity is completely attenuated in GHSR KO mice (Lim *et al.*, 2013). This confirms that a working ghrelinergic system is also required to elicit cannabinoid induced increases in hypothalamic AMPK (Lim *et al.*, 2013). The aforementioned experiments confirm a corroborative interaction, which in part relies on shared activation of AMPK signalling cascades, between cannabinoid and ghrelin pathways in stimulating feeding within the HYP.

Rational for Present Thesis

There are several pieces of evidence that lead to the formulation of our hypothesis that ghrelin and endocannabinoid systems likely interact within the VTA to drive feeding and more specifically that ghrelin's orexigenic effects within this region depend on a functional endocannabinoid system. First, there is evidence that these two systems interact within the HYP to promote feeding, as genetic and pharmacological blockage of either endocannabinoid or ghrelin signalling is sufficient to attenuate increases in food intake seen when either CB-1R or GHSR agonists are administered within the HYP of WT animals (Tucci *et al.*, 2004; Kola *et al.*, 2005; Kola *et al.*, 2008; Lim *et al.*, 2013). Second, GHSRs and CB-1Rs are found within brain regions of the mesolimbic dopamine system including the VTA (Herkenham *et al.*, 1991; Guan *et al.*, 1997). Lastly, both ghrelinergic and endocannabinoid systems are known to stimulate non-homeostatic feeding primarily by activating neurons within the mesolimbic dopamine system (e.g. VTA) (Abizaid *et al.*, 2006; Kirkham *et al.*, 2002; Szazbo *et al.*, 2002; Melis *et al.*, 2004).

In order to test if a functional endocannabinoid system is required to mediate ghrelin's orexigenic effects within the VTA we pre-treated rats with peripheral rimonabant and monitored food intake after administering ghrelin into the VTA (30 minutes after IP rimonabant). We predicted that by pre-treating rats with peripheral rimonabant we would be able to attenuate increases in food intake traditionally seen when ghrelin is microinjected into the VTA (Abizaid *et al.*, 2006). Furthermore, we proposed that if the interaction of these two systems is essential to the modulation of feeding in the VTA, as it is in the HYP, then the disruption of the ghrelin system should induce changes in the endocannabinoid system (i.e. changes in CB-1R expression) within this region as well as other important feeding related brain regions (i.e. HIP, NA, PFC, and HYP). To address this hypothesis we examined CB-1R mRNA expression levels, within the aforementioned regions, of ghrelin receptor knockout (GHSR KO) relative to wild-type rats *via* RT qPCR experiments.

General Methods

Animals

The male Long-Evans rats (300-500 grams) that were used in the food intake study were purchased from Charles River (St. Constant, Quebec, Canada). The Fawn-hooded hypertensive GHSR KO and WT rats used for RT qPCR were bred at the Carleton University animal care facility but were originally purchased from Transposagen Biopharmaceuticals Incorporated (Lexington, KY, USA). Upon delivery, rats from the food intake study were housed individually; whereas, those used in the RT-qPCR study were housed in pairs following weaning. In both cases rats were housed in plexiglass cages (48 cm x 26 cm x 20 cm) with access to ad libitum chow (Harlan

Diets; 3.3 kcal.g⁻¹; 4% fat; 48% carbohydrates; 14.3% protein) and water. Delivered rats were given 1 week to habituate to Carleton University animal care facilities, which are temperature (22°C) and humidity (45-55%) controlled. The animals were handled regularly throughout this period to minimize stress during experimental manipulations. All procedures followed Carleton University Animal Care Committee guidelines of the Canadian Council on Animal Care (CCAC) under p13-04 & p10-39 protocols.

Intra-VTA Cannula Surgical Procedures

Rats were subjected to an isoflurane:oxygen (5:2) mixture to induce anesthesia; however, they were switched to a 2.5:2 mix throughout the procedure to maintain anesthesia. Likewise, the rats were given a 1 mg/kg dose of Metacam (5 mg/ml) IP on the day of surgery to provide additional operative analgesia. Similarly, the rats received Metacam (5 mg/ml) at a dose of 0.5 mg/kg for at least 2 days following surgery to maintain post-operative analgesia. The rats head's were shaved prior to placement into the stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Tear gel was administered to mitigate dehydration of the rats' eyes. Their scalp was cleaned with germistat, alcohol, and proviodine to ensure that surfaces surrounding the incision site would remain aseptic. An incision was made along the midline, from just anterior to bregma to just posterior of lambda, and the skin was pinned back using hemostats to provide ample room to measure and mark out the anterior to posterior (AP = -5.3 mm) and medial to lateral (ML= +2.0 mm) coordinates of the VTA (from bregma). A mechanical surgical drill was used to remove the skull in the location that the unilateral guide cannula was later inserted. Likewise, the drill was exploited to make three other holes that were used to

implant screws that were later used to anchor the cannula. The cannula was inserted to the appropriate depth (DV= -7.6 mm) on an 8° tilt to avoid hitting the aqueduct. Using the three jeweller's screws and dental cement the cannula was secured to the skull. To prevent clogging of the guide cannula, a dummy cannula of the same length as the guide cannula was inserted into the stabilized guide. The incision was then sutured shut with PROLENE monofilament sutures and the rats were taken off of the anesthetic and placed in freshly clean cages. The rats' behavior and wounds were monitored closely for at least 1 hour post surgery for signs of complications. Post surgery rats were checked at minimum 3 times daily for a week following surgery to ensure optimal recovery before they were allowed to take part in experiment 1.

Histological Analysis and Cannula Placement Verification

Following the food intake study, rats were quickly decapitated, and their brains were collected and post-fixed in 4% paraformaldehyde (PFA) for 72 hours. Following this 72 hour period, the 4% PFA was removed and replaced with a 30% sucrose solution dissolved in 0.1 M phosphate buffer. The brains were stored at 4°C for approximately 48 hours or until the brains had sunk to the bottom of the vial. The brains were removed from the 30% sucrose solution and placed onto a metal cutting plate where the anterior part of the brain was cleaved off to facilitate preparation of the mid-posterior brain fragment, containing the VTA, for cannula verification placements. These midbrain sections were sliced on a LEICA CM1900 cryostat into 50 µm thick coronal sections. The location of the tip of the cannula in each rat brain was scored and recorded, with tracks not within the vicinity of the VTA being excluded from further analysis (see Figure 3). The anterior portion of the brain was kept for future NA c-FOS studies.

Figure 3: Summary of cannula placements for experiment 1. The purple dots represent those placements that were deemed to fall in an appropriate location within or just above the VTA and therefore were included in the statistical analysis. The red dots represent cannula which did not fall within the vicinity of the VTA and were therefore excluded from analysis.

Reverse Transcription Quantitative PCR

Rats were quickly decapitated, their brains were extracted, and the desired brain regions were punched (i.e. VTA, HIP, NA, PFC, and HYP) into 500 µls of TriZol (Invitrogen) and stored at -80 °C until the day of RNA extraction. On the day of RNA extraction the punches were homogenized & were then left to incubate for 5 minutes at 15-30°C to ensure all nucleoprotein complexes had time to dissociate prior to the addition of chloroform (0.1 ml). The samples were agitated for 15 seconds and incubated at 15-30°C for a further 2-3 minutes before the samples were spun at 12 000 x g for 15 minutes at 2-8°C to separate the desired aqueous phase. The RNA was precipitated from the aqueous phase by mixing it with 0.25 mls of isopropyl alcohol and 6.5 µls of linear acrylamide (co-precipitate) for 10 minutes (15-30°C). The samples were then subjected to a 12 000 x g centrifugation at 2-8°C. The RNA pellets were washed twice with 75% ethanol, spun down at 7 500 x g, and were dissolved in 15 µls of RNAase free water. The quality of the extracted RNA was assessed using a Thermo Scientific Nanodrop 100 spectrophotometer by comparing the ratio of the absorbancies at 260 nm over 280 nm (ratio close to 2 was considered ideal). Likewise, the concentration of the RNA was also determined using the Nanodrop spectrophotometer. The extracted RNA was reversed transcribed into cDNA by first adding 1 µL of oligo (dT) primer (Invitrogen) to 9 µls of a RNA and diethylpyrocarbonate (DEPC) water solution (ensuring 5000 ng of RNA from each sample was present in the reaction) and heating it for 5 minutes at 70°C. A master mix containing the following components per sample was made: 4 µLs of 5X first strand buffer (Invitrogen), 2 µLs of 0.1 M dithiothreitol (Invitrogen), 1 µL of RNase inhibitor (Promega), 1 µL of 10 mM deoxynucleotide triphosphate (Invitrogen), 1 µL of DEPC water and 1 µL of SSII RT (Invitrogen).

10 µls of this master mix was added to each of the samples. The samples were run on a PTC-200 Thermal Cycler (MJ Research) at 42°C for 1.5 hours followed by a ten minute 90°C incubation. The cDNA was diluted to a final concentration of 0.75 ng/µl and was stored at -20 °C until RT-qPCR experiments commenced. The sequences of the primers used for the amplification of the CB-1R, and housekeeping genes β-actin & glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: CB-1R forward-5'-CTGGTTCTGATCCTGGTGGT-3 (Lundanyi *et al.*, 2008); CB-1R reverse- 5- TGTCTCAGGTCCTTGCT CCT-3 (Lundanyi *et al.*, 2008); β-actin forward: 5'-TCATGAAG TGTGACGTTGACATCCG-3'; β-actin reverse: 5'-CCTAGAAGCATTGCGGTGCACGATG-3'; GAPDH forward: 5'-AAGATGGTGAAGGTCGG TGT-3'; GAPDH reverse: 5'-CTTGCCGTGGTAGAGTCAT-3'. In short, 5 µls of each cDNA sample was added to each well of the PCR plate before 2 µls of working primer solution (2 µM), 3 µls of MilliQ H₂O, and 10 µls of iQ SYBR Green Super Mix combined with fluorescein (BioRad) were added. All samples along with non-template controls were run in duplicate using primers for the CB-1R and housekeeping genes GAPDH and β-actin. The PCR plate was run on a MyIQ Single Color RT-qPCR Detection System (Bio-Rad). The program used was as follows: 30 second 95°C setting followed by 45 cycles of denaturing (10 seconds at 95°C), annealing (30 seconds at 55°C), and extension phases (20 seconds at 72°C). The program concluded with a 1 minute 95°C step proceeded by a 1 minute 55°C step. The primers used to investigate CB-1R and housekeeping gene expression were tested for their amplifications efficiencies using a standard curve method. Only those primers which fell between 90-110% efficient were kept for RT-qPCR experiments. The RT-qPCR data was analyzed using the $2^{-\Delta\Delta C_t}$ method (previously described in Schmittgen & Livak, 2008). This method is a comparative method to present gene expression of a treatment condition or genotype (i.e.

GHSR KO) relative to controls (i.e. WT). This method also normalizes the data to appropriate control housekeeping genes to ensure that changes in target gene expression are reflective of actual alterations in expression levels between two different groups and are not reflective of discrepancies in quality or concentration inherent in the cDNA samples. The housekeeping genes, GAPDH and β -actin, were used to express the relative CB-1R mRNA levels in accordance with the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

Food intake and locomotor activity data from experiment 1 were each analyzed using mixed repeated measure analysis of variance (ANOVA) where the within subjects factor was time and the between subject factor was the treatment (IP saline/intra-VTA saline, IP saline/intra-VTA ghrelin, IP rimonabant/intra-VTA saline, and IP rimonabant/intra-VTA ghrelin). In both instances, Mauchly's test of sphericity was violated and thus the Greenhouse-Geisser correction was used to adjust the degree's of freedom and compensate for these violations (all p values reflect this correction). Analyses of the mean cumulative food intake and locomotor activity between groups at each time point was also analyzed *via* univariate ANOVAs. LSD post hoc tests were performed when significant main effects were found. The $2^{-\Delta\Delta C_t}$ values computed following RT-qPCR experiments were analyzed using independent samples t-tests where the grouping variable was the genotype (GHSR KO vs WT) and the test variable was the $2^{-\Delta\Delta C_t}$ value.

Experiment 1: Rimonabant Peripheral Injections Attenuate the Orexigenic Effect of Ghrelin

Infused into the VTA

Method

This study was designed to test whether a functional endocannabinoid system is important for modulating ghrelin's orexigenic effects within the VTA. We were interested if pharmacological blockage of CB-1R, *via* peripheral injections of rimonabant, could dampen or eliminate the traditional increase in food intake seen with ghrelin infusions within the VTA.

To this end, forty Long-Evans rats were given *ad libitum* access to normal chow and water for 1 week before surgery to establish a pre-surgery baseline. The animals were then subjected to the VTA cannula surgical procedure and were given 1 week to recover. On post surgery day 7, half the rats received IP injections of rimonabant (1.0 mg/kg) while the other half received 0.9 % saline. Thirty minutes following the IP injection, half the rats were given intra-VTA infusions (i.e. microinjections) of ghrelin (1 μ g in 0.5 μ l over 1 minute); while, the other half received saline infusions (refer to Figure 4). Injections were completed on or around noon therefore rats were not in their active feeding period nor were they in a fasted state. All microinjections were completed using a GenieTouch™ dual syringe pump (Kent Scientific Corporation). This design yielded 10 rats per group for the following four treatments: IP saline/intra-VTA saline, IP saline/intra-VTA ghrelin, IP rimonabant/intra-VTA saline, and IP rimonabant/intra-VTA ghrelin. Food intake was monitored 1, 2, 4, & 6 hours post micro-infusions. Additionally, locomotor activity was also monitored for the first four hours post-microinjection to ensure that any differences in food intake were not a result of changes in locomotor activity. A week later these injections were repeated, albeit in a counter-balanced

fashion. However, this time the rats' food intake and locomotor activity were not measured and the rats were sacrificed 1 hour after receiving their microinjection. The animals were rapidly decapitated and their brains were post-fixed for cannula verifications and future NA c-FOS experiments.

Figure 4: Depiction of experiment 1 design. This study included the following 4 treatment groups: IP vehicle/intra-VTA saline, IP vehicle/intra-VTA ghrelin, IP rimonabant/intra-VTA saline, and IP rimonabant/intra-VTA ghrelin. Note that rimonabant (1.0 mg/kg) or vehicle was given intraperitoneal 30 minutes before ghrelin (1 μ g) or saline was infused (1 μ l over 1 minute) into the VTA *via* microinjection. Injections were given on both day 7 and day 14 albeit in a counter balanced fashion.

Experiment 2: Comparing CB-1R mRNA Expression within Important Feeding Nuclei of GHSR

KO and WT Rats

Method

In this study, we investigated relative levels of CB-1R mRNA in GHSR KO and WT rats in different feeding related brain regions (i.e. VTA, HIP, NA, PFC, and HYP). The rationale behind this experiment was to see if disrupting ghrelin signalling induces alterations in the endocannabinoid system, particularly with respect to the expression of CB-1Rs in these brain regions. 12 GHSR KO rats and 8 WT rats were sacrificed, their brains were removed and punches of the feeding related regions were processed according to the RT-qPCR methods section.

Results

IP Rimonabant Attenuates the Orexigenic Effect of Ghrelin Infused into the VTA

To investigate if a functional endocannabinoid system is required to mediate ghrelin's orexigenic effects within the VTA the following treatments were administered: IP vehicle/intra-VTA saline, IP vehicle/intra-VTA ghrelin, IP rimonabant/intra-VTA saline, IP rimonabant/intra-VTA ghrelin and food intake was subsequently measured at several time points (1, 2, 4, & 6 hrs). For these treatments, rimonabant was administered IP at a dose of 1.0 mg/kg; whereas, 1 µg of ghrelin was dissolved in 0.5 µl of saline and infused into the VTA over a 1 minute time period for the intra-VTA ghrelin microinjections.

Figure 5 illustrates the mean cumulative food intake of each of the four groups measured 1, 2, 4, and 6 hours after their corresponding microinjections. A repeated measures ANOVA revealed that all of the treatment groups ate significantly more over the 6 hour time period (main effect for time, $F_{(1.879, 37.589)} = 18.668$, $p < .05$). Likewise, the mean cumulative food intake between the treatment groups changed differentially over time (i.e. significant time and treatment interaction) as assessed by testing within subject effects using a repeated measures ANOVA ($F_{(5.63, 37.589)} = 2.502$, $p < .05$). LSD post hoc tests showed that those animals that were not pretreated with rimonabant but did receive intra-VTA ghrelin (i.e. IP vehicle/intra VTA ghrelin) ate significantly more than the other 3 groups ($p < .05$). Moreover, the increased food intake effect observed following intra-VTA ghrelin infusion was completely attenuated in rats that were pre-treated with rimonabant (i.e. IP rimonabant/intra-VTA ghrelin); however, rimonabant alone (i.e. IP rimonabant/intra-VTA saline) did not significantly reduce feeding as none of the three aforementioned groups (i.e. IP vehicle/intra-VTA saline, IP rimonabant/intra-

VTA saline, & IP rimonabant/intra-VTA ghrelin) differed from each other in terms of their mean cumulative food take (collapsed across time) ($p > .05$).

Analyses of the mean cumulative food intake between groups at each time point (i.e. 1, 2, 4, 6 hours) were compared *via* univariate ANOVAs. An ANOVA on the mean cumulative food intake 1 hour after microinjection detected no significant between subject effects ($F_{(3, 20)} = 2.064, p > .05$). Rats that were administered IP vehicle/intra-VTA ghrelin had the highest food intake at this time point; however, the effect did not attain significance.

Univariate ANOVA on the mean cumulative food intake 2 hours after microinjection revealed significant between treatment effects ($F_{(3, 20)} = 4.454, p < .05$). Additional LSD post hoc tests demonstrated that rats within the IP vehicle/intra-VTA ghrelin group consumed significantly more standard chow on average than the other three groups ($p < .05$)

Similarly, the mean cumulative food intake between the treatment groups significantly differed 4 hours after microinjection ($F_{(3, 20)} = 3.057, p = .052$). LSD post hoc tests highlighted that the IP vehicle/intra-VTA ghrelin group ate significantly more than IP vehicle/intra-VTA saline ($p < .05$) & IP rimonabant/intra-VTA ghrelin ($p < .05$) groups but not more than those rats which received the IP rimonabant/intra-VTA saline treatment ($p > .05$).

Lastly, significant between treatment effects remained 6 hours post microinjection ($F_{(3, 20)} = 3.668, p < .05$). The rats that received ghrelin into the VTA but were not treated with rimonabant (i.e. IP vehicle/intra-VTA ghrelin) continued to have significantly higher mean cumulative food intake compared to the saline control (i.e. IP vehicle/intra-VTA saline) ($p < .05$) and those pretreated with rimonabant (i.e. IP rimonabant/intra-VTA ghrelin, IP rimonabant/intra-VTA saline) ($p = .05$).

Figure 5: Mean cumulative food intake ($\pm$ S.E.M) of non-fasted male rats during their light cycle receiving one of the following four treatments: IP vehicle (Veh)/ Intra-VTA saline (Sal) ($n = 4$), IP rimonabant (Rim)/Intra-VTA Sal ($n = 7$), IP Veh/Intra-VTA ghrelin (Ghr) ($n = 7$), or IP Rim/Intra-VTA Ghr ($n = 6$). Food intake was measured 1, 2, 4, & 6 hours after each rat's microinjection. IP injections were given 30 minutes before microinjections. Note that rimonabant and ghrelin were administered at 1.0 mg/kg and 1 μ g/ μ l respectfully. * $p < .05$.

The Ability of Rimonabant to Attenuate the Orexigenic Effects of Ghrelin in the VTA is Independent of Locomotor Activity

To ensure that the changes in food intake observed were due to the corresponding treatments and not due to increased or decreased locomotor activity, the rats' ambulatory activity, measured by total number of beam breaks, was measured in the time between their IP injections and microinjections (i.e. -30 minutes) as well as 30, 60, 120, & 240 minutes proceeding their microinjections (see Figure 6). A repeated measures ANOVA determined that there was no significant difference in locomotor activity between the four treatment groups across time (i.e. no time and treatment interaction) ($F_{(3.658, 24.386)} = 2.01, p > .05$). Furthermore, separate analyses of the mean horizontal activity count between groups at each time point were compared *via* univariate ANOVAs. Again no significant differences in locomotor activity were revealed between the four treatment groups in between the two injections (i.e.-30 min) ($F_{(3, 20)} = 0.073, p > .05$) or at 30 ($F_{(3, 20)} = 1.377, p > .05$), 60 ($F_{(3, 20)} = 1.395, p > .05$), 120 ($F_{(3, 20)} = 1.860, p > .05$), and 240 ($F_{(3, 20)} = 1.999, p > .05$) minutes post microinjection.

Figure 6: Mean horizontal activity count ($\pm$ S.E.M) of non-fasted male rats during their light cycle receiving one of the following four treatments: IP Veh/ Intra-VTA Sal ($n = 4$), IP Rim/Intra-VTA Sal ($n = 7$), IP Veh/Intra-VTA Ghr ($n = 7$), or IP Rim/Intra-VTA Ghr ($n = 6$). IP injections were given 30 minutes before microinjections. Note that rimonabant and ghrelin were administered at 1.0 mg/kg and 1 μ g/ μ l respectively. There was no significant ($p > .05$) difference in locomotor activity between any of the treatment groups at any of the time points.

GHSR KO Rats Have Significantly Lower VTA but Higher PFC CB-1R mRNA Expression than WT

Rats

In experiment 2, RT-qPCR was used to investigate the relative expression of CB-1R mRNA within GHSR KO relative to WT rats in the following five feeding associated brain regions: VTA, HIP, HYP, NA, & PFC. Independent-samples t-tests were conducted to compare the expression of CB-1R mRNA, reflected by $2^{-\Delta\Delta CT}$ values, in GHSR KO versus WT rats.

GHSR KO rats ($M = 0.59$, $SEM \pm 0.11$) had significantly ($t(18) = 2.48$, $p = .014$) lower VTA CB-1R mRNA expression than corresponding WT rats ($M = 1.06$, $SEM \pm 0.20$) (see Figure 7). In contrast, GHSR KO rats ($M = 1.65$, $SEM \pm 0.23$) had significantly ($t(17) = -2.03$, $p = .059$) higher PFC CB-1R mRNA expression compared to WT rats ($M = 1.05$, $SEM \pm 0.13$) (see Figure 8). There was no significant difference ($t(14) = 0.63$, $p = .950$) in CB-1R mRNA expression between GHSR KO ($M = 1.98$, $SEM \pm 0.27$) and WT ($M = 2.03$, $SEM \pm 1.00$) rats with respect to the HYP (see Figure 9). Likewise, no significant difference ($t(15) = -.560$, $p = 0.584$) within the NA was found between GHSR KOs ($M = 1.37$, $SEM \pm 0.19$) and WTs ($M = 1.18$, $SEM \pm 0.32$) (see Figure 10). Lastly, there was also no significant difference ($t(16) = -1.03$, $p = .319$) in CB-1R mRNA expression between GHSR KO ($M = 1.41$, $SEM \pm 0.12$) and WT ($M = 1.14$, $SEM \pm 0.25$) rats within the HIP (see Figure 11).

Figure 7: Relative CB-1R mRNA expression ($\pm$ S.E.M) within the VTA of WT ($n = 8$) and GHSR KO ($n = 12$) rats analyzed using $2^{-\Delta\Delta C_t}$ method (previously described in, (Schmittgen & Livak, 2008)). GHSR KOs had significantly lower mean VTA CB-1R mRNA expression than corresponding WT rats ($p = .014$).

Figure 8: Relative CB-1R mRNA expression ($\pm$ S.E.M) within the PFC of WT ($n = 8$) and GHSR KO ($n = 11$) rats analyzed using $2^{-\Delta\Delta C_t}$ method (previously described in, (Schmittgen & Livak, 2008)). GHSR KOs had significantly higher mean PFC CB-1R mRNA expression than corresponding WT rats ($p = .059$).

Figure 9: Relative CB-1R mRNA expression ($\pm$ S.E.M) within the hypothalamus of WT ($n = 6$) and GHSR KO ($n = 10$) rats analyzed using $2^{-\Delta\Delta Ct}$ method (previously described in, (Schmittgen & Livak, 2008)). There was no significant difference between WT and GHSR KO rats ($p > .05$).

Figure 10: Relative CB-1R mRNA expression ($\pm$ SEM) within the nucleus accumbens of WT ($n = 7$) and GHSR KO ($n = 10$) rats analyzed using $2^{-\Delta\Delta C_t}$ method (previously described in, (Schmittgen & Livak, 2008)). There was no significant difference between WT and GHSR KO rats ($p > .05$).

Figure 11: Relative CB-1R mRNA expression ($\pm$ SEM) within the hippocampus of WT ($n = 8$) and GHSR KO ($n = 10$) rats analyzed using $2^{-\Delta\Delta C_t}$ method (previously described in, (Schmittgen & Livak, 2008)). There was no significant difference between WT and GHSR KO rats ($p > .05$).

Discussion

This current thesis aimed to test the hypothesis that the orexigenic effects of ghrelin in the VTA depend on a functional endocannabinoid system. Several findings led to the development of the above hypothesis. First, studies have shown that both ghrelin and endocannabinoid systems are important for modulating homeostatic feeding within the hypothalamus (Tucci *et al.*, 2004; Kola *et al.*, 2008). Second, hypothalamic disruption of either one of these systems (i.e. genetic or pharmacological) has been shown to attenuate or completely eliminate the orexigenic effects of the other system's appetite stimulating compound (i.e. ghrelin or endocannabinoids) (Tucci *et al.*, 2004; Kola *et al.*, 2005; Kola *et al.*, 2008; Lim *et al.*, 2013). Third, GHSRs and CB-1Rs are both known to be expressed within the VTA (Herkenham *et al.*, 1991; Guan *et al.*, 1997). Lastly, several autonomous research groups have demonstrated that stimulation of either ghrelinergic or endocannabinoid systems within the VTA increases food intake primarily through the activation (direct and indirect) of dopamine neurons that originate in this area (Abizaid *et al.*, 2006, Kirkham *et al.*, 2002; Szazbo *et al.*, 2002; Melis *et al.*, 2004; Sinnayah, 2008).

Data from experiment 1 show that, as expected, rats given IP vehicle/intra-VTA ghrelin injections consumed more food than control rats over a period of 6 hours post infusion. This group ate a majority of this food within the first hour as they consumed on average 1.5- 2 grams more than all other groups. Furthermore, ghrelin continued to elicit increased feeding during the next hour (2 hours post microinjection) as the IP vehicle/intra-VTA ghrelin group ate a further 0.5- 1 gram more standard chow than all other treatments. Ghrelin's orexigenic effect tailed off 2 hours post microinjection as increases in food intake between groups and in general

was minimal (4 & 6 hours after microinjection); however, the cumulative food intake of the IP vehicle/intra-VTA ghrelin group remained significantly higher than controls up to 6 hours after the microinjection. This supports previous findings showing that ghrelin can stimulate food intake when infused into the VTA and that ghrelin increases food intake most substantially within the first 2 hours (Naleid *et al.*, 2005; Abizaid *et al.*, 2006; King *et al.*, 2011). Ghrelin's ability to enhance feeding within this region relies in part on its capacity to increase the firing rate of GHSR-positive dopamine neurons that originate in the VTA (Abizaid *et al.*, 2006). Interestingly, ghrelin has been shown to stimulate these VTA dopamine neurons both directly by binding to GHSR receptors located on their surface and indirectly by increasing the number of excitatory synapses contacting them (Abizaid *et al.*, 2006). Once activated these GHSR-positive VTA dopamine neurons, which project to regions such as the PFC, HIP, & NA, are thought to increase dopamine turnover in target areas to enhance feeding (Abizaid *et al.*, 2006).

The orexigenic effects induced by intra-VTA ghrelin are dependent on a functional endocannabinoid system as pre-treatment with a sub-anorectic dose of rimonabant before administration of intra-VTA ghrelin completely attenuated feeding to a level indistinguishable from controls at all time points tested. Importantly, rimonabant alone (IP rimonabant/intra-VTA saline) did not diminish feeding beyond controls (IP vehicle/intra-VTA saline) validating that the rimonabant dose used was sub-anorectic (i.e. unable to reduce standard chow intake when administered alone). The fact that a very small dose of rimonabant that did not affect feeding on its own completely eliminated ghrelin's orexigenic effects when infused into the VTA suggests that rimonabant's likely interfered with ghrelin's ability to stimulate VTA dopamine

neurons. This hypothesis draws support from previous intra-tegmental rimonabant experiments demonstrating that rimonabant dampens dopamine neurotransmission in the nucleus accumbens core and behaviorally reduces food self-administration (Oleson *et al.*, 2012). Consistent with this, it is reasonable to presume that the reduced food intake observed in the rats that were pre-treated with rimonabant (IP rimonabant/intra-VTA ghrelin) relative to those that were only administered intra-VTA ghrelin (IP vehicle/intra-VTA ghrelin) could be the result of an overall suppression of VTA dopamine neuron activity in the former. Mechanistically, one of the ways in which this could be the case is if the inhibitory inputs (i.e. GABA) synapsing on VTA dopamine neurons are in general more numerous than their corresponding excitatory input counterparts (i.e. glutamate afferents). A majority of the non-dopaminergic neurons within the VTA are GABAergic and the abundance of GABA synapses onto VTA dopamine neurons has been reported by a plethora of autonomous research groups (Bayer & Pickle, 1991; Johnson & North 1992; Charara *et al.*, 1996; White 1996; Steffensen *et al.*, 1998; Carr & Sesack, 2000; Omelchenko & Sesack, 2009). Consequently, rimonabant pretreatment would mitigate the binding of endocannabinoids to their pre-synaptically located receptors on both GABA and glutamate axons to prevent inhibition of GABA and glutamate release; however, due to the increased prevalence of GABA inputs or stronger GABAergic connections, the main effect would be a heightened inhibitory tone and an overall hyperpolarization of VTA dopamine neurons (Figure 12, panel A). This would effectively counter the ability of ghrelin to stimulate VTA dopamine neurons and could be responsible for the depressed feeding observed in the IP rimonabant/intra-VTA ghrelin group relative to those that were only treated with ghrelin (i.e. IP vehicle/intra-VTA ghrelin). Furthermore, GABAergic neurons within the VTA may send inhibitory

synapses onto glutamate excitatory inputs that are contacting VTA dopamine neurons (Omelchenko & Sesack, 2009; Ciccarelli *et al.*, 2012). Therefore with rimonabant pretreatment, the GABA neurons would become disinhibited (reduced CB-1R activation) and would reduce the overall excitatory tone produced by glutamate neurons (less numerous). This would effectively mitigate the ability of ghrelin to excite VTA dopamine neurons (Figure 12, panel B). Lastly, recent studies have also localized CB-1Rs on astrocytes (Navarrete & Araque, 2008; Navarrete & Araque, 2010). When treated with CB-1R agonists, these neurons release glutamate to facilitate excitation and potentiate synaptic plasticity (Navarrete & Araque, 2008; Navarrete & Araque, 2010; Castillo *et al.*, 2012). Therefore the interruption of astrocyte CB-1R activation, which normally potentiates synaptic transmission onto VTA dopamine neurons, may be one of the ways in which rimonabant blocks ghrelin's orexigenic effects when ghrelin is infused into the VTA (Figure 12, panel C). A combination of the aforementioned proposed mechanisms may be responsible for the ablation of ghrelin's orexigenic effects following rimonabant pre-treatment. Future electron microscopy studies that allow for the quantification of CB-1R-positive (immunostaining for CB-1R) asymmetric and symmetric synapses onto VTA dopamine neurons, in both GHSR KO and WT rodents, will provide valuable information about how ghrelin and endocannabinoids alter VTA dopamine neuron activation by modifying inputs into the hub of the mesolimbic dopamine system.

Figure 12: Hypothetical mechanisms in which rimonabant treatment within the VTA could counteract ghrelin's stimulation of VTA dopamine neurons. (A) Higher GABAergic compared to glutamatergic presence. (B) Inhibition of glutamate excitatory release onto VTA dopamine neurons by GABAergic neurons. (C) Blockage of CB-1Rs on astrocytes to prevent glutamate release.

Alternatively, it is plausible that rimonabant blockage of CB-1Rs elsewhere in the brain contributed to the attenuation of the feeding effects induced by intra-VTA ghrelin. Future experiments, in which rimonabant is infused into the VTA before intra-VTA ghrelin, may help elucidate whether blockage of CB-1Rs specifically within the VTA is sufficient to mitigate ghrelin's orexigenic effects within this region.

Measuring the food intake and locomotor activity of the 4 treatment groups concurrently was an important precautionary measure to rule out the possibility that any feeding effects induced by rimonabant or ghrelin were due to modifications in locomotor activity. The cumulative horizontal activity analysis revealed no significant differences in locomotor activity between the 4 treatment groups at any of the tested time points. It seems clear that ghrelin's ability to increase food intake when infused into the VTA was not a result of an overall increase in locomotor activity as reflected by the normal locomotor activity measured in the IP vehicle/intra-VTA ghrelin group. Similarly rimonabant's ability to ablate ghrelin's appetitive effects was not caused by decreased locomotor activity within the IP rimonabant/intra-VTA ghrelin group.

It is evident from experiment 1 and previous studies that ghrelin and endocannabinoid systems are important and interact to encourage feeding behaviours within multiple important feeding related brain regions (i.e. VTA, HIP, NA, PFC, and HYP); however, the nature of this interaction and whether the mechanism differs between brain regions remain unclear (Tucci *et al.*, 2004; Kola *et al.*, 2008; Isokawa, 2012; Sarvari *et al.*, 2014). For instance, within the hypothalamus, it has been speculated that CB-1Rs and GHSRs may heterodimerize to increase the activation of AMPK signalling cascades, which then leads to the stimulation of feeding (Kola *et al.*, 2008, Lim *et al.*, 2013); whereas, within the VTA, the mechanisms in which endocannabinoid and ghrelin systems interact to stimulate feeding are less clear since CB-1Rs are not found on GHSR positive dopamine neurons, whose activity has been shown to drive food intake (Herkenham *et al.*, 1991; Matsuda *et al.*, 1993; Guan *et al.*, 1997; Abizaid *et al.*, 2006). To this end, we decided to examine CB-1R expression within the VTA, regions that the

VTA projects to such as the PFC, HIP, & NA, and other important feeding nuclei such as the HYP, in rats with deletions to the GHSR compared to WT rats. It was hypothesized that if the alleged interaction between these two systems is truly essential to the modulation of feeding in the VTA then a disrupted ghrelin signalling system should induce changes in the endocannabinoid system that ought to be reflected by modifications in CB-1R expression in the aforementioned associated brain regions. Findings from experiment 2 highlighted that CB-1R mRNA is decreased within the VTA, increased within the PFC, and unchanged in the HYP, NA, and HIP of GHSR KO compared to WT rats.

When making inferences about mRNA expression data, particularly with respect to CB-1R expression, one must take into consideration multiple factors. First, the endocannabinoid system is extremely complex and seems to function diversely in different locations within the body. For example, endocannabinoids are thought to activate AMPK activity in the hypothalamus, decrease AMPK activity in the heart, and although they mostly act as retrograde messengers within the brain they also have been shown to have autocrine capabilities (Kola *et al.*, 2005, Jantti *et al.*, 2013). Second, CB-1Rs are almost exclusively found on axon terminals; whereas, CB-1R mRNA is traditionally found in neuron cell bodies. Therefore one must be careful in interpreting results based on either mRNA or protein expression alone (spatially separated). Third, there have been conflicting results in terms of whether CB-1R agonists (e.g. 2-AG, AEA, & THC) decrease or increase the transcription of the CB-1R gene (Laprairie *et al.*, 2012). Within numerous brain regions, many studies report that CB-1R density is inversely related to ligand availability such that chronic CB-1R agonist treatment (i.e. THC) results in decreased CB-1R mRNA levels (i.e. desensitization) (Harrold *et al.*, 2002; Breivogel *et al.*, 1999;

Corchero *et al.*, 1999). Intriguingly, many of these studies also found regions in which chronic CB-1R agonist treatment resulted in an upregulation of CB-1R mRNA; although, these regions often conflicted with those reported from similar studies (Romero *et al.*, 1996; Zhuang *et al.*, 1998; Breivogel *et al.*, 1999; Corchero *et al.*, 1999; Harrold *et al.*, 2002). Conversely, many cell culture conducted studies using neural and non-neural cell types demonstrated a substantial increase in CB-1R mRNA expression upon endogenous ligand (i.e. 2-AG & AEA) administration (Mukhopadhyay *et al.*, 2010; Proto *et al.*, 2011; Borner *et al.*, 2007; Laprairie, *et al.*, 2013). It seems as though when CB-1R positive cells are isolated and treated with CB-1R agonist, as is the case with *in vitro* studies, they respond by upregulating CB-1Rs (i.e. CB-1R mRNA); however, CB-1R positive neurons *in vivo* may differentially regulate their CB-1Rs depending on the duration of CB-1R agonist treatment (chronic versus acute) and/or based on external influence (e.g. afferent signals, local neural microenvironments). Future gas chromatography (GC) flame ionization detection and GC-mass spectroscopy studies, that will allow for the quantification of 2-AG and AEA within the aforementioned feeding related regions, in which CB-1R mRNA expression data was collected, will be instrumental in confirming the relationship between endocannabinoid tone and CB-1R mRNA expression.

The lower CB-1R mRNA expression observed within the VTA of GHSR KO rats relative to their WT counterparts is likely a result of a lower local endocannabinoid tone within this region. Consistent with this, ghrelin has an extensive role in activating VTA dopamine neurons (both directly and indirectly) within WT rats; however, within GHSR KOs, ghrelin's ability to excite these neurons would be lost (Abizaid *et al.*, 2006). It follows that the VTA dopamine neurons found in the GHSR KOs would presumably be less excitable (i.e. decreased burst firing

frequencies) and would have less retrograde endocannabinoid messenger release compared to corresponding WT rats. Consequently, this would reduce the demand of CB-1Rs within this region and may help explain why CB-1R mRNA expression is lower in the GHSR KO relative to the WTs. The downregulation of CB-1R mRNA in response to this chronic lowered endocannabinoid tone cannot be explained by changes in CB-1R expression within a majority of the afferent connections to the VTA as the cell bodies of the neurons that project to this region are found elsewhere (Lupica & Riegel, 2005). Similarly, it cannot reflect changes in CB-1R mRNA within VTA dopamine neurons themselves as studies using radiolabeled CB-1R agonists have failed to localize CB-1Rs on VTA dopamine neurons (Herkenham *et al.*, 1991). Consequently, the lowered CB-1R mRNA expression observed in the GHSR KOs is likely reflecting a downregulation of CB-1Rs within the other predominant subset of neurons found within the VTA; GABAergic interneurons and efferents that originate in the VTA and which are known to project to the NA, PFC, and the VTA itself. Future studies investigating the excitability of VTA dopamine neurons within GHSR KO rats relative to WT rats, especially after manipulation of endocannabinoid system elements (i.e. CB-1R agonist & antagonist), will provide a more complete understanding of how these two systems interact within the VTA.

The mechanisms underlying the upregulation of CB-1R mRNA within the PFC of GHSR KO compared to WT rats are unknown as the relationship between ghrelin and endocannabinoid systems within this region is still not well understood; however, recent evidence suggests that these two systems do interact within this region. Functional magnetic resonance imaging blood oxygen level dependant (BOLD) techniques detect a significant alteration (i.e. decrease) in the BOLD response in the PFC of anaesthetized rats in response to ghrelin (Logothetis *et al.*, 2001;

Sarvari *et al.*, 2014). Interestingly, this decrease is partially attenuated by pre-treating rats with rimonabant; illustrating that the endocannabinoid system either directly or indirectly works in tandem with the ghrelin system to modulate neuronal activity within the PFC (Sarvari *et al.*, 2014). Immunofluorescence studies using antibodies specific for the CB-1R and the GHSR may provide a better anatomical picture of the distribution of these receptors within the PFC and may provide leads as to how these two systems integrate their signals within this region.

Interestingly, the CB-1R mRNA expression profile of the GHSR KO rats relative to the WT rats (i.e. low VTA expression and high PFC) mimic CB-1R mRNA expression and binding density patterns observed in both human subjects with psychological disorders and in rodents that have been subjected to chronic stress paradigms (Hungund *et al.*, 2004; Dean *et al.*, 2001; Dalton *et al.*, 2011; Hill *et al.*, 2008; Bortolato *et al.*, 2007). For example, CB-1R mRNA is upregulated in the PFC of depressed suicide victims and patients who have been diagnosed with paranoid schizophrenia (Hungund *et al.*, 2004; Dean *et al.*, 2001; Dalton *et al.*, 2011). Moreover, chronic unpredictable stress and chronic mild stress paradigms cause a significant increase in CB-1R receptor binding site density and mRNA within the PFC, yet a significant decrease in these measures within the VTA (Hill *et al.*, 2008; Bortolato *et al.*, 2007). There has not been an extensive amount of work done in terms of characterising the genotypic and corresponding functional or behavioural differences of GHSR KO relative to WT rodents. The finding that the GHSR KO rats used have the same dysregulated CB-1R mRNA expression profile (within the PFC and VTA) of chronically stressed mice and human subjects with physiological disorders may help explain the observed predisposition towards anxiety and depressive related

pathologies reported previously (Lutter *et al.*, 2008; Patterson *et al.*, 2010; Patterson *et al.*, 2013).

The fact that there was no significant difference in CB-1R mRNA expression within the hypothalamus of GHSR KO compared to WT rats was quite surprising given the known interdependence of these two systems within this region (Tucci *et al.*, 2004; Kola *et al.*, 2005; Kola *et al.*, 2008; Lim *et al.*, 2013). One might predict that CB-1R mRNA expression would be upregulated within GHSR KO relative to WT rats due to potential compensatory effects as substantial evidence has demonstrated that both GHSR and CB-1R activation contribute to the stimulation of AMPK signalling cascades within the hypothalamus (see Figure 1). Moreover, it has been predicted that these two receptors (i.e. GHSR & CB-1R) may form heterodimers to increase their agonistic activity within this region as studies have shown that both CB-1Rs and GHSRs do form heterodimers with other receptors to enhance their agonistic activity (Ellis *et al.*, 2006; Turu & Hunyady, 2010; Schellekens *et al.*, 2013; Alen *et al.*, 2013; Lim *et al.*, 2013). Accordingly, if the ghrelin receptor is defective then any activity that normally would occur as a result of the GHSR/CB-1R heterodimer would be ablated and presumably elicit changes in CB-1R expression. The fact that there was no detectable difference in hypothalamic CB-1R mRNA levels between the two genotypes could have been the result of many different phenomena. First, the hypothalamus has many small and functionally diverse nuclei that contain numerous different cell types (some of which have antagonistic roles) (e.g. arcuate nucleus) (Abizaid & Horvath, 2008; Sanchez-Lasheras *et al.*, 2010). Consequently, when the hypothalamus was punched CB-1R mRNA levels within different nuclei and even within opposing cell types could have offset any changes that may have existed within specific subnuclei or even a particular

subset of neurons within a given nucleus. Laser collection microdissection, a technique that uses a microscope to visualize and software to select a specific nuclei or even single cells of interest before the region of interest is separated and collected from surrounding tissue, *via* a laser, may prove to be invaluable for analysing CB-1R mRNA expression within distinct nuclei and sub-nuclei of the hypothalamus. The feasibility of this technique for the aforementioned purpose was recently demonstrated when dopamine 2 receptors and G-protein-regulated inward-rectifier potassium channel 2 mRNA were quantified via RTq-PCR after dopamine neurons from mice and human samples were laser dissected and collected for analysis (Dragicevic *et al.*, 2014). Alternatively, it is conceivable that GHSR ablation does significantly affect the endocannabinoid system (i.e. less or more CB-1R protein expression) within the hypothalamus but that it is not reflected in basal CB-1R mRNA expression. There are many processes in between when a gene is transcribed, translated into a protein, and then transported to its active location (e.g. general translation, folding, post-translational modifications, stability, degradation, and membrane localization); many of which could be differentially regulated in the GHSR KO relative to WT rats. Lastly, since we were comparing hypothalamic CB-1R mRNA within a genetic KO model instead of pharmacologically blocking GHSR signalling, it could be that other systems, rather than the endocannabinoid system, compensated for the lack of ghrelin signalling and hence no major observable differences in CB-1R mRNA manifested.

Similarly, although there is appreciable expression of GHSR mRNA and CB-1R binding density (as determined using radio labeled CB-1R agonist) within the HIP and NA of rats (Herkenham *et al.*, 1991; Guan *et al.*, 1997), RT qPCR analysis revealed that CB-1R mRNA levels

were unchanged within these regions in the GHSR KO compared to the WT rats. It was surprising to see no differences within the HIP given the reported importance of the interaction between ghrelin and endocannabinoids in modulating synaptic plasticity and neurogenesis associated with learning and memory (Cuellar & Isokawa, 2011; Isokawa, 2012; Cahill *et al.*, 2014). Similarly, it was unexpected to see comparable CB-1R expression within the NA of both genotypes as rat fMRI BOLD studies suggest that ghrelin and endocannabinoid systems strongly affect one another within the NA (Sarvari, *et al.*, 2014). The potential reasons listed above for the lack of effect seen in the hypothalamus may be applicable to describe the analogous absence of major changes observed in the HIP and NA; however, other non-discussed mechanisms may also contribute.

Future studies will be needed to elucidate why no significant CB-1R mRNA changes are observed within the HYP, HIP, & NA of GHSR KOs relative to WTs even though significant evidence suggests that these two systems are intimately interconnected within these regions (Tucci *et al.*, 2004; Kola *et al.*, 2005; Kola *et al.*, 2008; Lim *et al.*, 2013; Cuellar & Isokawa, 2011; Isokawa, 2012; Sarvari, *et al.*, 2014). Furthermore, figuring out how endocannabinoid levels and other factors modify CB-1R mRNA expression under different conditions (chronic, acute) and how these factors ultimately pertain to functional levels of CB-1Rs on the surface of cells would facilitate the elucidation of molecular circuits and allow for more accurate predictions of the role endocannabinoids play in various nuclei.

Conclusions

Here we demonstrated that non-fasted rats will significantly increase their consumption of standard chow following intra-VTA infusions of ghrelin and that this corresponding increase in food intake depends on a functional endocannabinoid system as peripheral pre-treatment with rimonabant completely attenuated the enhanced feeding to control levels. Together, this data suggests that ghrelin targets the VTA to increase food intake through a mechanism that requires a functional endocannabinoid system. Furthermore, we also demonstrated that CB-1R mRNA expression is significantly reduced in the VTA but enhanced in the PFC of GHSR KO relative to WT rats. Interestingly, this may reflect a previously uncharacterized genotypic vulnerability to psychological related disorders as this same CB-1R mRNA expression profile has been commonly observed in subjects with physiological disorders as well as chronically stressed rodents. Lastly, the CB-1R mRNA study also demonstrated that disruption of the ghrelin system induces changes in the endocannabinoid system differentially within distinct brain regions. This highlights the need for the elucidation of the exact nature of the interaction between these two systems, within each specific nucleus, so that future pharmaceuticals can be more appropriately designed and their mechanisms better understood.

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