

IMPACT OF GHRELIN RECEPTOR ANTAGONISM
ON NICOTINE AND COCAINE DRUG REACTIVITY IN RATS

A Dissertation

by

PATRICK SHANE CLIFFORD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,
Committee Members,

Head of Department,

Paul Wellman
Shoshana Eitan
Gerald Frye
Carnel Morgan
Paul Wellman

May 2013

Major Subject: Psychology

Copyright 2013 Patrick Shane Clifford

ABSTRACT

Ghrelin is a 28 amino acid peptide that interacts with ghrelin receptors (GHS-Rs) to modulate brain reinforcement circuits. Systemic ghrelin infusions augment cocaine (COC) stimulated locomotion and conditioned place preference (CPP) in rats, whereas genetic or pharmacological ablation of GHS-Rs has been shown to attenuate the acute locomotor-enhancing effects of nicotine (NIC) and COC, and to blunt the CPP induced by food, alcohol, amphetamine and COC in mice. The stimulant NIC can induce CPP and like COC, repeated administration of NIC induces locomotor sensitization in rats. In experiment 1, we examined the effects of GHS-R antagonism with JMV 2959 on COC-induced locomotion and found that JMV 2959 suppresses COC-induced locomotor sensitization. In experiment 2, we examined the effects of GHS-R antagonism with JMV 2959 on NIC-induced locomotion and found that JMV 2959 suppresses NIC-induced locomotor sensitization. In experiment 3, we examined the effects of GHS-R knockout on COC-induced locomotion and found that animals sustaining GHS-R knockout display a suppression of COC-induced locomotor sensitization. In experiment 4, we examined the effects of GHS-R knockout on COC-induced locomotion and found that animals sustaining GHS-R knockout display a suppression of COC-induced locomotor sensitization. In experiment 5, we examined the effects of JMV 2959 on NIC-enhanced intracranial self-stimulation (ICSS) responding and found that JMV 2959 alone had no effect, but when combined with NIC, JMV 2959 pretreatment reversed the enhancement of responding produced by NIC. In experiment 6, we examined the effects of GHS-R knockout on ICSS responding and found that animals sustaining GHS-R knockout were unable to acquire ICSS at current intensity levels that

would support responding by WT animals. It was not until the intensity was ramped up four fold that these knockout rats were able to acquire responding. These results show that antagonism of GHS-Rs diminishes the reinforcing effects of NIC and COC. This provides evidence that antagonists of GHS-Rs could be useful in the treatment of drug addiction, particularly that involving nicotine.

ACKNOWLEDGEMENTS

First, I would like to thank my committee chair, Paul Wellman, for all of the technical skills he has taught me over the years and for all of the advice he has given me. If it wasn't for his mentoring, I would never have made it to this stage in my life. You never got upset with me even with all the mistakes I made, which helped me learn from them instead of agonizing over them. You have been a mentor in more than just research and I doubt I will ever feel as at home as I did working with you in your lab.

I would also like to thank my committee members, Shoshana Eitan, Gerald Frye, and Caurnel Morgan, for being patient with me throughout this process and for their assistance in formulating and constructing the experiments presented in this dissertation. I want to thank the people that helped me perform these experiments and keep me focused: Juan Rodriguez, Shawn Bates, Destri Schul, Sam Hughes, Tracey Kniffin, Jeff Thompson, and Sam Buckman. Finally, I would like to thank my wife, Mila Clifford, for sticking with me and helping me survive through this ordeal and my dad, Kevin Clifford, for the support he has given me all these years.

NOMENCLATURE

ACTH.....	adrenocorticotrophic hormone
ARC	arcuate nucleus
COC	cocaine
CPP	conditioned place preference
DA.....	dopamine
GHS-R.....	growth hormone secretagogue receptor, ghrelin receptor
GHS-R ^(-/-)	ghrelin receptor knockout
GOAT	ghrelin o-acyltransferase
ICSS	intracranial self-stimulation
ICV.....	intracerebroventricular
i.m.	intra-muscular
i.p.	intra-peritoneal
MFB	medial forebrain bundle
NAC	nucleus accumbens
NIC.....	nicotine
NPY.....	neuropeptide Y
POMC	proopiomelanocortin
PVN.....	paraventricular nucleus of the hypothalamus
VTA	ventral tegmental area
WT	wild type

TABLE OF CONTENTS

	Page
ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	iv
NOMENCLATURE.....	v
TABLE OF CONTENTS.....	vi
LIST OF FIGURES.....	viii
 CHAPTER	
I INTRODUCTION.....	1
II GENERAL METHODS.....	23
Subjects.....	23
Housing.....	23
Drugs.....	24
Surgical Procedures.....	24
Histological Procedures.....	25
Apparatus.....	26
III EXPERIMENT 1: EFFECT OF JMV 2959 ON COCAINE-INDUCED LOCOMOTOR SENSITIZATION.....	27
Background.....	27
Experimental Procedures.....	29
Results.....	32
Discussion.....	33
IV EXPERIMENT 2: EFFECT OF JMV 2959 ON NICOTINE-INDUCED LOCOMOTOR SENSITIZATION.....	35
Background.....	35
Experimental Procedures.....	36
Results.....	38
Discussion.....	40

CHAPTER	Page
V	EXPERIMENT 3: EFFECT OF GHRELIN RECEPTOR KNOCKOUT ON COCAINE-INDUCED LOCOMOTOR SENSITIZATION..... 43
	Background..... 43
	Experimental Procedures 44
	Results..... 46
	Discussion..... 47
VI	EXPERIMENT 4: EFFECT OF GHRELIN RECEPTOR KNOCKOUT ON NICOTINE-INDUCED LOCOMOTOR SENSITIZATION 50
	Background..... 50
	Experimental Procedures 50
	Results..... 52
	Discussion..... 54
VII	EXPERIMENT 5: EFFECT OF JMV 2959 ON NICOTINE-ENHANCED INTRACRANIAL SELF-STIMULATION..... 55
	Background..... 55
	Experimental Procedures 58
	Results..... 61
	Discussion..... 62
VIII	EXPERIMENT 6: EFFECT OF GHRELIN RECEPTOR KNOCKOUT ON INTRACRANIAL SELF-STIMULATION..... 63
	Background..... 63
	Experimental Procedures 63
	Results..... 66
	Discussion..... 67
IX	GENERAL DISCUSSION AND CONCLUSIONS..... 70
	REFERENCES 77

LIST OF FIGURES

FIGURE		Page
1	Structure of ghrelin	5
2	VTA – NAcc pathway	7
3	Structure of JMV 2959.....	17
4	Behavioral analysis of Experiment 1	29
5	Impact of 6 mg/kg JMV 2959 on cocaine locomotor sensitization	31
6	Behavioral analysis of Experiment 2	36
7	Impact of 3 and 6 mg/kg JMV 2959 on nicotine locomotor sensitization.....	38
8	Behavioral analysis of Experiment 3	44
9	Impact of GHS-R KO on cocaine locomotor sensitization.....	46
10	Changes in food intake in WT and GHS-R ^(-/-) rats after ghrelin.....	48
11	Behavioral analysis of Experiment 4	51
12	Impact of GHS-R KO on nicotine locomotor sensitization	53
13	Theoretical ICSS construct	56
14	Impact of JMV 2959 and cocaine on rate-frequency responding in ICSS	57
15	Behavioral analysis of Experiment 5	59
16	Impact of JMV 2959 and nicotine on rate-frequency responding in ICSS....	61
17	Behavioral analysis of Experiment 6	64
18	Typical ICSS electrode placements	65
19	Impact of GHS-R KO on rate-frequency responding in ICSS.....	66
20	Simplified GHS-R functionality circuit.....	71

CHAPTER I

INTRODUCTION*

In recent years, a significant amount of research has been dedicated to finding a solution to the problem of obesity. One of the major health concerns in the United States as well as other Western European societies is obesity. The prevalence of obesity is increasing (Olshansky et al., 2005). By the year 2035, if the current progression continues, it is estimated that some 90% of Americans will be obese (Garko, 2011). One line of obesity-related research has focused on the factors that stimulate eating (Wren et al., 2000), while another has focused on the factors that inhibit eating. From this research came the discovery of ghrelin, a 28 amino acid peptide secreted from the stomach and gut. Initially, ghrelin was found to be an endogenous ligand for the growth-hormone secretagogue receptors (GHS-Rs), which are responsible for releasing growth-hormone from the pituitary (Kojima et al., 1999, Kojima and Kangawa, 2005). It wasn't long after ghrelin's discovery that ghrelin was detected in a large number of other areas in the body and that it plays a role in a considerable range of functions. It's important to note the fact that ghrelin is the only known peripheral peptide that stimulates food intake (Wren et al., 2000). From an obesity perspective, treatments that diminish ghrelin function might be useful for reducing food intake.

Although ghrelin was originally identified in the stomach, it was also later detected in a number of brain regions (Kojima et al., 1999). Ghrelin acts on GHS-Rs which are also found in numerous areas of the body and several areas of the brain. The principal areas

* Part of this chapter is reprinted with permission from "Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fMRI and intracranial self-stimulation" Wellman, PJ, Clifford, PS, Rodriguez, JA, Hughes, S, Di Francesco, C, Melotto, S, Tessari, M, Corsi, M, Bifone, A, Gozzi, A, 2011. *Addiction Biology*, 17(5):908-19, Copyright 2011 by Wiley.

where GHS-Rs have been localized are along the vagus nerve and in brain areas that include the ventral tegmental area (VTA), hippocampus, arcuate nucleus (ARC) of the hypothalamus, nucleus accumbens (NAC), amygdala, and even the Edinger-Westphal nucleus which lies just dorsal to the VTA (Kaur and Ryabinin, 2010). Systemic ghrelin is thus positioned to alter vagus nerve signaling to the brain and to enter the brain to act on GHS-Rs.

As mentioned earlier, ghrelin binds to GHS-Rs to release growth-hormone from the pituitary, but ghrelin and GHS-Rs have also been associated with several other functions. Endogenous ghrelin plays an important role in stimulating food intake and inducing growth (Wren et al., 2000, Wren et al., 2001a, Depoortere, 2009). In addition to systemic ghrelin, intracerebroventricular (ICV) injections of ghrelin significantly increase food intake (Nakazato et al., 2001). Along with growth induction and feeding behavior, ghrelin has been implicated in memory retention and anxiety (Carlini et al., 2002). Recently, there have been studies determining that ghrelin plays a role in metabolic control and that the peptide increases gastric emptying (Tschop et al., 2001). Ghrelin also acts through GHS-Rs to moderate the stress response and to modulate energy homeostasis (Abizaid et al., 2006a).

The observation that ghrelin induces eating led to a search for GHS-R antagonists that might be of use to decrease food intake as a potential means to fight obesity. However, ghrelin is currently being studied in a variety of ways, by itself, and in combination with drugs of abuse for its role in mediating reward. There are two different methods of approach to investigating the impact of ghrelin on reward: ghrelin activation, and ghrelin system inactivation. Within these approaches, there lies a wide range of methods which themselves also employ an array of techniques.

Ghrelin administration increases motivation to acquire preferred foods (Fulton, 2010) while decreasing water intake (Hashimoto and Ueta, 2011). In locomotor tests, administration of ghrelin has been found to slightly increase locomotion and to enhance the hyperactivity produced by psycho-stimulants such as cocaine (COC) (Wellman et al., 2005). Ghrelin administration alone and coupled with psycho-stimulants have both been shown to increase accumbal dopamine (DA) overflow (Jerlhag et al., 2006). Also, ghrelin acts to facilitate food-induced conditioned place preference (CPP) and even low ghrelin doses augment COC-generated CPP (Davis et al., 2007, Perello et al., 2010).

While little work has been done with ghrelin and addictive drug self-administration, serum levels of ghrelin have been shown to rise preceding periods of reinstatement for COC (Tessari et al., 2007). Surprisingly, in an intracranial self-stimulation (ICSS) task, wherein rats press a lever for a pleasurable electrical stimulation of their brain at varying frequencies, ghrelin decreases responding causing a rightward shift (unpublished observations) yet, GHS-R antagonists either do not change or increases responding producing a slight leftward shift (Wellman et al., 2012). Ghrelin administration, centrally or peripherally, has also been shown to produce wakefulness and arousal (Korotkova et al., 2006), as well as having the capacity to alter circadian rhythm (Yannielli et al., 2007).

In order to examine the effects of inactivation of ghrelin, researchers have used similar tests to the ones mentioned above for examining the effects of ghrelin administration. There are two main methods of inactivating the ghrelin system. The least complicated approach is through the use of GHS-R antagonists, predominantly a compound known as JMV 2959 (Moulin et al., 2007b, Salome et al., 2009b). The other primary method of inactivating the ghrelin system is to genetically knockout the production of ghrelin or to

knockout the development of GHS-Rs altogether (Zan et al., 2003). GHS-R knockout animals display reduced locomotor responses to COC (Abizaid et al., 2011). Since GHS-Rs have been found on the vagus nerve, one other avenue of research is to investigate the effects of vagotomy on the ghrelin system (Williams et al., 2003). Subdiaphragmatic vagotomy prevents the increase in ghrelin levels in response to food deprivation conditions. RNA silencing and immunosuppression of GHS-Rs are other viable techniques which have yet to be fully explored (Lu et al., 2009, Shrestha et al., 2009). Finally, it may be possible to block the formation of active ghrelin. Blockade of ghrelin o-acyltransferase (GOAT), which is required to form active ghrelin, may diminish circulating levels of the active form of this peptide (Takahashi et al., 2009). The term “ghrelin” refers to the octanoylated form of ghrelin (acyl ghrelin).

The GHS-R was first identified in 1996 and labeled as a pathway for controlling the release of growth hormone (Howard et al., 1996). These receptors, which are g-protein coupled receptors, react to small molecules called growth-hormone secretagogues (GHSs) and exert action via second messengers. As such, these receptors were understandably termed GHS receptors (GHS-Rs), which were recently designated more specifically as GHS-R1a. There is also a GHS-R1b, but this receptor hasn't been shown to react to ghrelin signaling (Howard et al., 1996). G-protein coupled receptors act by causing different downstream effects in various cellular systems when their ligands bind to them. In the case of GHS-Rs, the activity is believed to be through the G_q phospholipase C pathway where phospholipase C helps generate diacyl glycerol (DAG) and inositol triphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP2) (Holst et al., 2003, Holst et al., 2004). Acting as a second messenger, DAG activates Protein Kinase C (PKC). IP_3 induces phosphorylation

of some proteins (Alberts et al., 2002). Given that these effects ultimately increase calcium levels within neurons, it would not be surprising that activation of GHS-Rs is mostly excitatory (Takano et al., 2009) but in at least one system (dorsal raphe), ghrelin is inhibitory (Hansson et al., 2011).

It is interesting to note however, that at the time of the receptor's discovery the endogenous ligand was yet unknown. It was discovered a short time later that these identified receptors responded to a different unknown compound other than growth hormone releasing hormone which led to the later identification of the ghrelin peptide (Bennett et al., 1997).

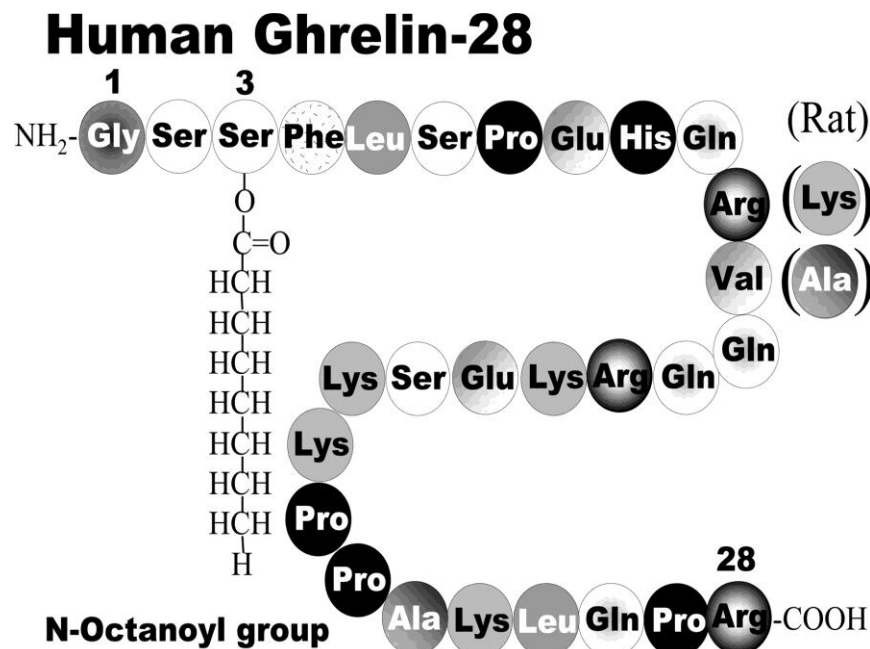


Figure 1. Structure of ghrelin (Kojima and Kangawa, 2005).

The ghrelin peptide was discovered by Kojima's group in 1999, while they were investigating the GHS-Rs in the pituitary that act to release growth hormone. This group was able to isolate and identify an endogenous ligand for these GHS-Rs. The newly identified peptide was named ghrelin, since the root of the word "ghre" means "grow" (Kojima et al., 1999). Ghrelin (see Figure 1) is composed of 28 amino acids with the octanoylation of the third serine, which is done by GOAT (Yang et al., 2008). This octanoylation is necessary to stimulate release of growth hormone since the acylated form is the most active. Human ghrelin is almost identical to the ghrelin found in rats, having only a two amino acid difference (Kojima et al., 1999).

Ghrelin is widely distributed throughout the body and the brain but is primarily secreted from the fundus of the stomach and is transported across the blood brain barrier and into the brain (Wren et al., 2001a, Banks et al., 2002). Although mostly identical to human ghrelin, mouse ghrelin differs in that it can only be transported across the blood brain barrier from blood to brain and not back across. This is likely due to the slight structural differences between human and mouse ghrelin. GHS-Rs are also distributed within the brain as well as in the peripheral nervous system (Wren et al., 2001a, Banks et al., 2002).

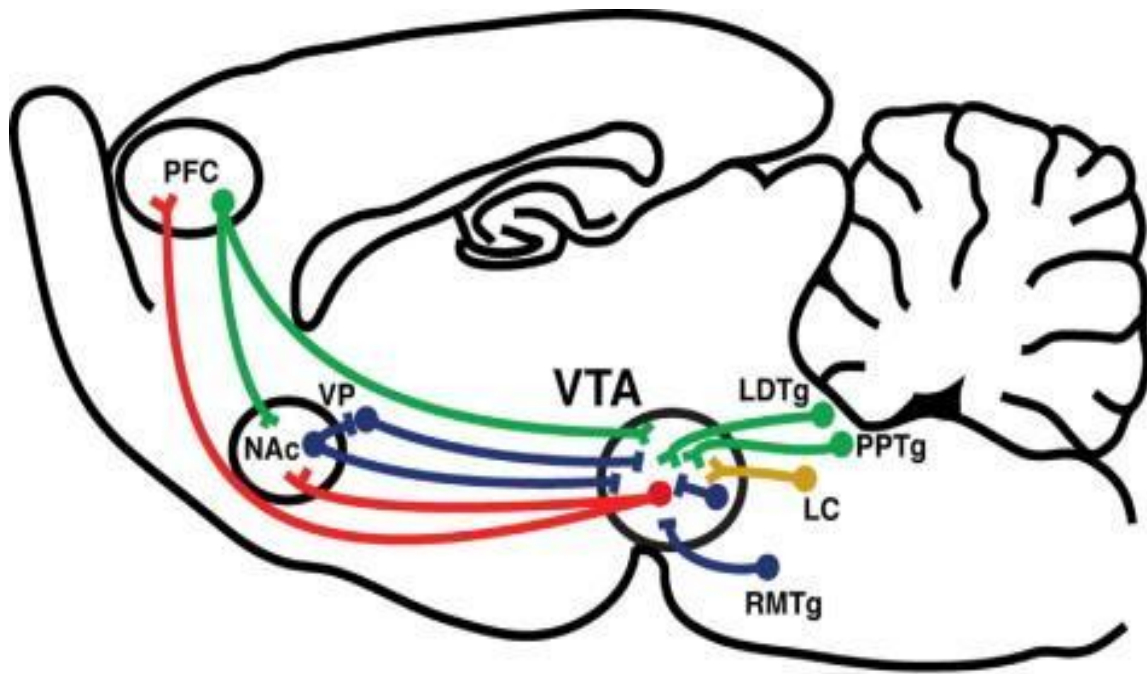


Figure 2. VTA – NAcc Pathway (Morikawa and Paladini, 2011). Red lines represent DA, green represent glutamate, blue represent GABA, and yellow represents norepinephrine.

The first GHS-Rs found were detected in the pituitary, as mentioned previously (Kojima et al., 1999). Subsequent studies reported receptors responding to ghrelin in the hippocampus and the ventral tegmental area (VTA) (Guan et al., 1997, Abizaid, 2009). In the VTA, ghrelin stimulates feeding behavior and may also affect metabolism. DA cells in the VTA also respond to insulin and leptin, which are well known metabolic hormones (Naleid et al., 2005, Abizaid, 2009, van Zessen et al., 2012). VTA neurons receive inputs from orexin neurons in the lateral hypothalamus (Richardson and Aston-Jones, 2012), which are also sensitive to ghrelin (see Figure 2). In the hippocampus, ghrelin promotes long-term potentiation (Diano et al., 2006, Banks et al., 2008) and increases memory retention as measured by increases in latency in a step-down passive avoidance of foot shock test. It can

also cause anxiety as shown by decreases in entries into open arms of the elevated plus maze and an induction of freezing (Carlini et al., 2002, Hansson et al., 2011).

Ghrelin and GHS-Rs have been identified in the ARC of the hypothalamus, where ghrelin triggers the release of peptides and neurotransmitters. This release influences food intake and plays a role in controlling energy homeostasis (Bagnasco et al., 2003, Cowley et al., 2003). Harrold et al. examined the location of GHS-Rs within the hypothalamus using immunohistochemistry to identify Fos expression linked to GHS-Rs. They found the ARC nucleus to be the area with the densest collection of GHS-Rs (Harrold et al., 2008). Ghrelin alters ARC inputs by augmenting neuropeptide Y (NPY) signaling, which stimulates food intake and ghrelin diminishes proopiomelanocortin (POMC) signaling. POMC signaling plays a role in the induction of satiety (Cowley et al., 2003). Systemic infusions of ghrelin leads to an increase in Fos in the ARC nucleus, which is expressed where neurons fire action potentials making Fos a marker for increases in neuronal activity (Scott et al., 2007). Ghrelin neurons have been located projecting from the hypothalamus to the brainstem, where they interact with the dorsal vagal complex. Ghrelin can act as a neurotransmitter, perhaps through stimulation of calcium signaling (Hou et al., 2006, Hori et al., 2008).

Ghrelin also acts on receptors in other areas of the brain, such as the NAC which is well known for playing a role in reward (Quarta et al., 2009). This area will be more important later in the review when ghrelin and reward is addressed directly. Ghrelin also binds to receptors in the amygdala, establishing a role for ghrelin in the emotional aspect of feeding and perhaps emotion per se (e.g. fear and anxiety) (Malik et al., 2008). GHS-Rs have also been identified in the Edinger-Westphal nucleus, and ghrelin within this region

may play a role in the facilitation of alcohol consumption (Zigman et al., 2006, Kaur and Ryabinin, 2010).

Ghrelin has been shown to play a role in a wide variety of processes throughout the body. Ghrelin binds to GHS-Rs in the ARC nucleus and the hypothalamus to stimulate secretion of growth hormone (Wren et al., 2000, Mano-Otagiri et al., 2006). Intraperitoneal (i.p.) administration of 30 nMol ghrelin increases growth hormone levels, and ICV administration of 2 nMol ghrelin causes release of adrenocorticotrophic hormone (ACTH) (Wren et al., 2000).

It has been well established that ghrelin is a systemic peptide and the only one known to increase food intake. For example, chronic ghrelin administration induces overeating and can lead to obesity when animals are fed a high fat, diet while antagonism of GHS-Rs reduces gastric emptying and leads to losses of body weight (Asakawa et al., 2003). Endogenous ghrelin levels rise and fall in a diurnal pattern reaching their peaks immediately before dark and light periods when gastric content is at its lowest (Murakami et al., 2002). Ghrelin levels rise in the absence of food and fall following feeding when on a free feeding schedule, but these fluctuations can be altered by inducing “set” meal times. Anticipation of a large meal, like that seen in human feeding patterns, results in higher peak levels of ghrelin prior to feeding (Drazen et al., 2006). Higher acyl ghrelin levels have been associated with faster gastric emptying, perhaps through facilitation of gastric motor function (Tschop et al., 2001). Des-acyl ghrelin, the inactive form of ghrelin, has been shown to decrease the rate of gastric emptying and patients suffering from gastrointestinal disorders have displayed lower acyl ghrelin levels than do healthy patients (Ogiso et al., 2011).

Most studies that have examined the biological function of ghrelin have done so by examining the effects of administration of exogenous ghrelin. In Siberian hamsters, systemic administration of ghrelin induces the same increases in food hoarding and foraging as seen under periods of food deprivation while also stimulating food intake (Keen-Rhinehart and Bartness, 2005). This is interesting because food deprivation results in very small increases in food intake or results most often in no increase at all in hamsters (Keen-Rhinehart and Bartness, 2005). Systemic (i.p.) administration of 6 nMol ghrelin induces an increase in food seeking behaviors similar to that seen in animals following 24 hour food deprivation (Davidson et al., 2005).

One way that ghrelin has been found to induce stimulation of feeding is by phosphorylation of cAMP response element-binding protein (CREB) to inhibit the effect of cholecystokinin (CCK) which effects COC- and amphetamine-regulated transcript (CART) in vagal afferent neurons (de Lartigue et al., 2007). Peripheral ghrelin administration at 10 nMol causes an increase in Fos expression in the ARC nucleus, which can also be seen during periods of food deprivation suggesting that ghrelin is excitatory for eating (Ruter et al., 2003, Becskei et al., 2008). In addition to Fos induction within the ARC nucleus, peripheral ghrelin (10 nMol) administration increased Fos expression in the paraventricular nucleus of the hypothalamus (PVN) (Ruter et al., 2003). The PVN is a focal region for the induction of eating (Leibowitz, 1978). Injecting the unacylated form of ghrelin i.p., desacyl ghrelin, causes an increase in Fos expression in the ARC nucleus. Desacyl ghrelin also blocks the stimulatory effect on feeding of acyl ghrelin when administered simultaneously (Inhoff et al., 2008). This lends some evidence that Fos activation might not be related to the feeding effect of ghrelin. High plasma levels of desacyl ghrelin have been linked to

reductions of food intake and stimulation of adipogenesis (Inhoff et al., 2009). Ghrelin amplifies DA signaling in neurons expressing GHS-Rs and the DA D1 receptor subtype (Jiang et al., 2006). This will be important for understanding the biological function of ghrelin with regards to reward. Injection of ghrelin directly into the VTA and the NAC both result in stimulation of food intake. Ghrelin infusions into the VTA increase sucrose reward seeking, but injection into the NAC does not, suggesting the VTA is a locus for food motivation and reinforcing the notion that ghrelin acts on feeding through multiple pathways (Figlewicz and Sipols, 2010, Dickson et al., 2011, Skibicka and Dickson, 2011, Skibicka et al., 2011a).

ICV infusion at least 1 nMol ghrelin causes animals to increase their food intake (Nakazato et al., 2001). ICV administered ghrelin also results in an increased preference for high fat foods which leads to an increase in fat consumption (Shimbara et al., 2004). Chronic ICV administration of ghrelin leads to weight gain and adiposity (Wren et al., 2001b). ICV administration of ghrelin has also been known to cause increases in corticosterone levels and elevated body temperature (Jaszberenyi et al., 2006). Infusion of 1 nMol ghrelin directly into the third ventricle results in increases in food seeking behaviors as well (Davidson et al., 2005). Intra-third ventricular infusion of ghrelin increases food intake to a larger degree in already fat rats as opposed to lean rats and fat rats had significantly higher GHS-R mRNA present in the hypothalamus (Brown et al., 2007). When infused into the lateral ventricle or fourth ventricle, ghrelin stimulates food intake and increases expression of NPY mRNA (Kinzig et al., 2006, Spinedi et al., 2006) which suggests that ghrelin might act via NPY.

ICV infusion of 1.5 nMol ghrelin also results in increased memory retention and induction of anxiety evidenced by increases in freezing in an open field and reduction in the

number of entries into the open arms of an elevated plus maze (Carlini et al., 2002). Ghrelin promotes synapse formation in the hippocampus, which benefits spatial learning and memory and administration of ghrelin reverses the decreases in synapse density and impairments in memory observed following the ablation of the ghrelin gene (Diano et al., 2006). Infusion of 0.3 nMol and 3 nMol ghrelin directly into the hippocampus has been shown to improve memory consolidation (Carlini et al., 2010). These observations suggest that ghrelin may be useful in improving memory in elderly individuals. There is a possibility that ghrelin signaling may play a role in memory for food location, in behaviors such as foraging, for example.

Ghrelin can alter the function of multiple systems and multiple neurochemical pathways. ICV injection of ghrelin augments the release of norepinephrine and increases ACTH levels (Kawakami et al., 2008, Chuang and Zigman, 2010). Although infusion of ghrelin activates the stress pathway, ghrelin levels do not rise following exposure to an external stressor (Zimmermann et al., 2007). The ghrelin acylating enzyme, GOAT, has been implicated in glucose metabolism in that inhibition of GOAT prevents weight gain and lowers fat mass in mice on a high fat diet (Al Massadi et al., 2011). Ghrelin activates DA neurons that are responsible for regulation of homeostasis (Abizaid et al., 2006a, Palmiter, 2007, Abizaid and Horvath, 2008). Exogenous administration of 10 nMol ghrelin has also been shown to have a neuroprotective effect in the substantia nigra pars compacta, where neuronal loss is involved with the development of Parkinson's disease in mice (Andrews et al., 2009).

There are also some studies that show that ghrelin produces its effects through pathways other than DA. Some studies suggest ghrelin plays a role in the hypothalamic

pituitary system by activating the serotonin pathway, since previous research has shown ghrelin has the capacity to inhibit serotonin release in the hypothalamus (Brunetti et al., 2002, Jaszberenyi et al., 2006). Therefore, as ghrelin levels increase, the level of serotonin would then decline. It has been well established that serotonin inhibits food intake (Lam et al., 2010), so the implication of this observation is that ghrelin may increase food intake at least in part through inhibiting the release of serotonin. Ghrelin administration (3 nMol) into the cerebral ventricles has been shown to induce changes in emotional responses, specifically increases in anxiety and depression thought to be caused by moderation of serotonin signaling (Hansson et al., 2011). Another implication is that high levels of ghrelin may play a role in producing depression. Depression is thought to, at least in part, be a result of decreases in synaptic availability of serotonin. Since ghrelin inhibits the release of serotonin, higher levels of ghrelin might result in more severe cases of depression. Therefore, it may be interesting to know if anti-depressants would work well in people suffering from depression that have high levels of ghrelin or whether ghrelin antagonists would be useful as anti-depressant drugs.

It is readily accepted that COC stimulates locomotion. Acute systemic injection of ghrelin at a high enough dose to stimulate feeding (1 nMol) (Wren et al., 2001b) does not affect locomotion in rats by itself, but systemic ghrelin has been shown to augment the acute locomotor effects of COC in rats (Wellman et al., 2005). Food restriction has also been shown to augment psychostimulant action and up-regulate circulating levels of rat ghrelin. Repeated administration of feeding-relevant doses of ghrelin (5, 10 nMol) over a period of ten days induces a cross sensitization to COC, which augments COC stimulated locomotor responses (Wellman et al., 2008b). In other words, rats repeatedly exposed to ghrelin will

respond to their first exposure to COC with higher locomotor responses than do rats that were repeatedly exposed to vehicle. This could be indicative of how ghrelin is known to reorganize inputs in the reward pathway particularly in the VTA, to result in neural activation (sensitization) (Abizaid et al., 2006b). Injection of ghrelin directly into the VTA and the laterodorsal tegmental area result in an increase in locomotion and overflow of DA within the NAC (Jerlhag et al., 2007).

As mentioned before, ghrelin levels increase during periods of food restriction. Food restriction has been shown to augment psychostimulant induced CPP (Jerlhag et al., 2009). Administration of ghrelin augments food reward induced CPP in mice when using high fat foods (Perello et al., 2010). In mice, i.p. injection of 3 nMol ghrelin was sufficient to produce CPP by itself (Jerlhag, 2008). In rats, systemic administration of ghrelin prior to a CPP task augments the rewarding effects of COC, particularly in COC doses too low to induce a place preference by themselves (Davis et al., 2007). Interestingly, at usual higher doses of COC, ghrelin causes the opposite effect. What was found here was that the interaction of ghrelin and COC caused a leftward shift in the dose response curve for COC showing an augmentation of drug reward. What this means is that this leftward shift in the inverted U dose response curve causes lower doses of COC act as though they were significantly higher doses. Therefore, the highest dose tested appeared to be made aversive to the animals after ghrelin.

The usual standard for investigating addiction is using a task in which the reward is self-administered which is studied in multiple stages (Carroll et al., 2004). The first two stages are acquisition, which involves being conditioned to respond for a reward, and maintenance, which is demonstrated by continuing to respond for a reward in varying

conditions. Lastly is extinction, where responding is discontinued following absence of reward for responding, and reinstatement, where there is restoration of the drug seeking behavior sometimes due to exposure to the previous conditioned stimulus. Rats with higher serum ghrelin levels show higher incidence of reinstatement in COC self-administration after conditioned stimulus exposure compared to rats with lower ghrelin levels (Tessari et al., 2007). Self-administration of a reinforcer does not necessarily require exposure to drugs. ICSS tasks involve implanting electrodes into brain areas such as the medial forebrain bundle (MFB) and allowing responding to stimulate these areas with pleasurable pulses of electrical current (Olds and Milner, 1954). Paradoxically, systemic administration of 10 nMol ghrelin produces a dose-dependent rightward shift in responding and an increase in response threshold for the MFB stimulation. Systemic administration of COC produces the opposite effect, and ghrelin attenuates the COC shifts in responding when combining ghrelin and COC (Kniffin, unpublished data).

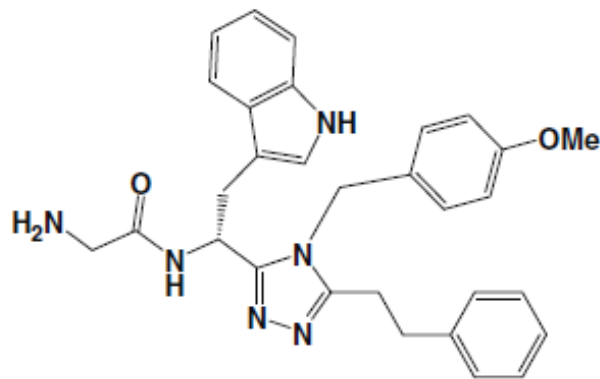
As mentioned before, ICV or intra-VTA infusions of ghrelin have been shown to increase DA levels up to as much as 130% of baseline in the NAC (Jerlhag et al., 2006, Kawahara et al., 2009). When ghrelin is administered peripherally, the change in DA overflow in the NAC is dependent on when the animal has last fed. Consumption of food results in the same increases in DA overflow that are seen when ghrelin is administered centrally, but removing access to food decreases DA levels in the NAC after systemic ghrelin administration (Kawahara et al., 2009). Ghrelin has also been shown to play a role in the regulation of arousal. ICV administration of ghrelin promotes wakefulness as do injections into the medial preoptic-area and the PVN (Szentirmai et al., 2007). Ghrelin has already

been shown to follow a circadian rhythm related to feeding times. Administration of ghrelin in to the suprachiasmatic nucleus can advance the circadian phase (Yannielli et al., 2007).

It is well established that ghrelin stimulates food intake, but few studies have observed the interactions of ghrelin and food reinforcement. Ghrelin administration has been shown to increase motivation to obtain food reward (Figlewicz and Sipols, 2010, Fulton, 2010). Infusion of ghrelin into the VTA causes rats to work harder to obtain food pellets than control rats (King et al., 2011). Ghrelin administration into the VTA also increases motivation to obtain a sucrose reward (Skibicka et al., 2011a). In addition to modulating food intake, systemic ghrelin has been shown to influence water consumption and centrally administered ghrelin inhibits water intake even while food was freely available during these water consumption tests (Mietlicki et al., 2009, Hashimoto and Ueta, 2011, Mietlicki and Daniels, 2011). The suppressive impact of ghrelin on water intake is worthy of comment since this result is largely unexpected. ICV infusion of ghrelin increases alcohol intake under free choice testing conditions (Jerlhag et al., 2009).

The other common approach to examining the role of ghrelin and reward is to inactivate ghrelin signaling. This can be accomplished in many ways, the first of which is through pharmacological antagonism of GHS-Rs. Before antagonists for the GHS-R were discovered, inverse agonists were used and were found to decrease the signaling of the GHS-Rs (Mietlicki and Daniels, 2011). Inverse agonists have negative efficacy and binding to receptors results in a response that is the opposite of what is produced by an agonist. Inverse agonists have not been explored in much detail due to the arrival of a mixed agonist or mixed antagonist, BIM-28163, a ghrelin analog that acts as an antagonist of the GHS-Rs. BIM-28163 blocks ghrelin induced growth hormone secretion, but it mimics ghrelin in its capacity

to stimulate food intake and increase weight gain (Halem et al., 2004, Moulin et al., 2007b). BIM-28163 reduces the ghrelin-induced Fos expression in the medial ARC nucleus but up-regulates Fos expression in the dorsal medial hypothalamus (Halem et al., 2005). Intra-VTA infusion of BIM-28163 blocks the appetitive effects of ghrelin and attenuates food intake following food deprivation (Abizaid et al., 2006b). Due to its varying effects on ghrelin signaling, BIM-28163 is not an ideal ghrelin antagonist. After BIM-28163, came the antagonist used most often in the current literature which has been labeled JMV2959 (Salome et al., 2009b).



JMV 2959
 IC_{50} (binding) = 32 nM
Antagonist

Figure 3. Structure of JMV 2959.

JMV2959 is a derivative of the triazole structure (Moulin et al., 2013) that (see Figure 3) shows low nanomolar affinity for GHS-Rs and is not a mixed agonist like BIM-28163 (Salome et al., 2009b, Wellman et al., 2012). ICV infusion of JMV2959 suppressed ghrelin induced food intake and blocks the increased food intake following periods of food deprivation (Bell et al., 1997, Wellman et al., 2012). Central administration of JMV2959 suppresses ghrelin induced increases in body weight and fat mass, and blocks the ghrelin induced decreases in energy use (Salome et al., 2009b).

Pharmacological antagonism of GHS-Rs has been shown to alter ghrelin signaling, and what is of interest is the interaction between ghrelin and reward. ICV or intra-tegmental ghrelin administration increases alcohol intake in a free choice test in mice, which is blocked with central or peripheral administration of JMV2959 (Jerlhag et al., 2009). These results may represent a food intake effect or a reward effect. JMV2959 also ablates CPP, DA release in the NAC, and locomotion increases following alcohol intake in mice (Jerlhag et al., 2009). Thus, ghrelin appears to alter ethanol reward, not simply ethanol's effect on feeding. Further, other drugs of abuse such as COC and amphetamine cause increases in accumbal DA release and increases in locomotion which can be blocked with JMV2959 (Jerlhag et al., 2010). Administration of JMV2959 peripherally blunts the ability of nicotine (NIC) to increase locomotion and DA release in the accumbens (Jerlhag and Engel, 2011).

One way of examining the effects of ghrelin that was touched upon earlier is to look at drug sensitization. It is well established that repeated administration of drugs of abuse can create sensitization such that each successive exposure to the drug results in an increased effect, compared to the previous one. It was mentioned earlier that repeated administration of ghrelin produces a cross sensitization to drugs of abuse, particularly COC (Wellman et al.,

2008b). This suggests that GHS-R activity is required for the induction of locomotor sensitization to COC. Similar to COC, NIC has the capacity to induce CPP and repeated administration of NIC induces locomotor sensitization.

Another way of examining the role of ghrelin in drug abuse via inactivation of ghrelin signaling is to genetically ablate the GHS-R or ghrelin product. One of the first studies to look at ghrelin knockout animals did so in mice and they were unable to find a difference between ghrelin knockout mice and wild type mice in regards to ghrelin stimulated feeding (Sun et al., 2003). However, ghrelin-knockout and GHS-R-knockout mice on restricted feeding schedules show reduced feeding compared to wild type controls (Abizaid et al., 2006b). Ghrelin-knockout and GHS-R-knockout mice also exhibit reduced blood glucose levels and respiration was mildly inhibited in the ghrelin-knockout mice (Sun et al., 2008).

As mentioned before, ghrelin seems to play a role in behaviors associated with anticipation of feeding. Animals sustaining genetic ablation of the GHS-R show attenuated meal anticipatory locomotion and attenuated Fos expression in the hypothalamus compared to wild type littermates (Blum et al., 2009). GHS-R (^{-/-}) mice show attenuated food anticipatory stimulated locomotion (LeSauter et al., 2009). Knockout of ghrelin in mice also decreases arousal and increases sleeping during the periods of light as well as decreases in sleep during periods of the dark (Szentirmai et al., 2009). GHS-R knockout mice also fail to develop a CPP to high fat diets as seen in wild type mice (Perello et al., 2010).

What is of interest here is the effect that genetic ablation of ghrelin or GHS-Rs has on the interaction of ghrelin and drug reward. As mentioned before, food restriction augments the behavioral and reinforcing effects of psychomotor stimulants such as COC possibly via increases in ghrelin levels. Under food restriction, GHS-R knockout mice do not

significantly increase anticipatory locomotor activity relative to wild types, but administration of COC at doses of at least 1.25 mg/kg is sufficient to increase locomotion in these ghrelin knock outs to a level equivalent to that seen in wild types (Clifford et al., 2011). Ghrelin knockout mice display a decrease in COC-induced stimulation of locomotion as well as ablation of DA changes in the striatum seen in wild type mice (Abizaid et al., 2011). The capacity of alcohol to increase DA release is blocked in ghrelin knockout mice and the alcohol induced locomotor increases are attenuated compared to wild type controls (Jerlhag et al., 2011).

GHS-R^(-/-) rats have been developed in Fawn Hooded Hypertensive (FHH) rats. The FHH-Ghsm1/Mcwi [GHS-R^(-/-)] strain was generated by the PhysGen Program in Genomic Applications by N-ethyl-N-nitrosourea (ENU) mutagenesis of Fawn Hooded Hypertensive (FHH) strain animals. Briefly, ENU-treated males were backcrossed and offspring were screened using a Targeting Induced Local Lesions in Genomes (TILLing) approach (Till et al., 2007). GHS-R-specific primers GHS-R_F: 5'- GTTTGTCAGTAGGCATGCAG -3' and GHS-R_R: 5'- GAAAGGCCATGTCTTAAGTTG -3' were used to screen for mutations in exon 2 of GHS-R (GenBank accession number NM_032075). The GHS-Rm1/Mcwi mutation was evident as a C>T transition of base pair of nucleotide 1027 of this sequence by Sanger sequencing, creating glutamine (CAG) to stop (TAG) codon change. This mutant animal was backcrossed and then intercrossed for more than 15 generations. Sanger sequencing was used to confirm the animals are homozygous.

While genetic ablation of GHS-Rs and pharmacological antagonism of GHS-Rs are the most common ways to examine inactivation of ghrelin signaling, there are some additional methods that need further exploration. As discussed earlier, there are GHS-Rs in

the vagus nerve and vagotomy is one approach to investigate ghrelin signal inactivation. Vagotomy does not have an effect on baseline levels of ghrelin, but it does prevent the rise in ghrelin seen after periods of food deprivation (Williams et al., 2003). The effect ghrelin has on food intake can also be blocked by immunosuppression. Acyl-ghrelin specific antibodies bind to acyl-ghrelin and inhibit calcium signals and ghrelin induced feeding increases in mice (Lu et al., 2009). Interfering with RNA to reduce ghrelin gene expression is another way ghrelin inactivation can be studied. This RNA interference lowers body weight and reduces blood ghrelin levels as much as 500 pg/ml (down from 2200 pg/ml) without having an impact on feeding (Shrestha et al., 2009). Polymorphisms of the genes that control production of either ghrelin or of ghrelin receptors might impair ghrelin function and may provide clues as to the functions of the ghrelin systems. Research shows that a polymorphism of the GHS-R has been linked to high alcohol consumption in human females and that high alcohol consumption can be suppressed with antagonism of GHS-Rs (Landgren et al., 2012). No research to date has linked such polymorphisms to either cocaine or nicotine addiction in humans.

Ghrelin seems to be involved in a number of important processes and being able to alter ghrelin signaling would be helpful in treating diseases and disorders associated with these functions, one example would be ghrelin vaccination (Zigman and Elmquist, 2006, Leite-Moreira and Soares, 2007). One vaccine study was done showing that rats administered a ghrelin vaccine slowed their weight gain and gained less body fat (Zorrilla et al., 2006). A GHS-R vaccine could potentially be used as well and administered ICV. Due to ghrelin's diverse effects, ghrelin signaling could be a useful avenue of therapy for obesity,

anorexia, gastric ulcers, and perhaps reproduction problems (Leite-Moreira and Soares, 2007).

In addition to ghrelin playing a role in drug abuse, drugs of abuse can play a role in altering ghrelin signaling. Ecstasy or 3,4-methylenedioxymethamphetamine (MDMA) causes acute increases in ghrelin levels which could explain changes in appetite (Kobeissy et al., 2008). It has been shown previously that increases in ghrelin activity causes increases in VTA activity, and the VTA has also been implicated in sexual reward (van Furth and van Ree, 1996). Increases in ghrelin levels in the VTA cause an increase in sexual reward. This opens up the possibility that ghrelin may play a role in the reinforcing properties of sexual behavior. Since MDMA causes an increase in ghrelin levels, it may exert at least part of its increase in sexual reward and sexual motivating effects through the ghrelin-VTA pathway. Ghrelin has also been implicated in sexual development, and ghrelin seems to play a role in the regulation of puberty (Repaci et al., 2011).

Based on the aforementioned literature, a logical step forward would be to examine the effects of modulation of ghrelin signal activity regarding drugs of abuse. The focus of the present experiments was to employ two distinct strategies to examine the role of GHS-Rs in drug-induced locomotor sensitization as well as shifts in ICSS response rates induced by COC or by NIC. The first strategy involved the use of JMV 2959 to antagonize GHS-Rs, while the second strategy was the genetic ablation of GHS-Rs in a genetic knockout rat.

CHAPTER II

GENERAL METHODS*

Subjects

Male Sprague-Dawley rats (Harlan Houston) and male Fawn Hooded Hypertensive (FHH) rats were used for the experiments. Age-matched parental FHH/EurMcwi strain males were provided as controls in our experiments. WT and GHS-R^(-/-) rats were held in quarantine for 30 days after arrival at TAMU. All rats were acclimated to the colony for a minimum of 7 days before the start of any experiment. All rats received food and water ad libitum and were housed on a 12:00 hour light/dark cycle with the lights on at 8:00am and off at 8:00pm. All procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals after receiving the approval of Texas A&M University's Institutional Animal Care and Use Committee.

Housing

Both the Sprague-Dawley and Fawn Hooded Hypertensive rats were single housed in standard polycarbonate cages with continuous access to food and water except as noted below. The colony room temperature was maintained at 21 + 1 ° C while the humidity was maintained at 60-70%.

* Part of this chapter is reprinted with permission from "Lobeline attenuates progressive ratio breakpoint scores for intracranial self-stimulation in rats" Wellman PJ, Elliott AE, Barbee S, Hollas CN, Clifford PS, Nation JR, 2008. *Physiology & Behavior* 93:952-957, Copyright 2007 by Elsevier.

Drugs

COC solutions were prepared by dissolving COC hydrochloride (a gift provided by Dr. Kevin Gormley of the Basic Research Division of NIDA) into 0.9% saline at concentrations of 10 mg/ml. COC dose was calculated as the salt. COC doses were chosen based on earlier studies involving locomotor sensitization (Miller et al., 1999). NIC was prepared by dissolving NIC hydrogen tartrate salt (HT: Sigma, St. Louis, MO, USA) into 0.9% saline at a concentration of 0.25 or 0.4 mg/ml, calculated as the free base. The pH of the NIC solution was adjusted to ~ 7.0 using sodium hydroxide. NIC doses were chosen based on other studies involving NIC induced locomotion (Bevins and Palmatier, 2003, Santos et al., 2009, Zago et al., 2012). The JMV 2959 hydrogen chloride was dissolved into 0.9% saline at a concentration of 3.0 and 6.0 mg/ml calculated as the salt and was administered in a volume of 1.0 ml/kg. The JMV 2959 was a kind gift from Jean-Alain Fehrentz of the Institut des Biomolécules Max Mousseron, Faculté de Pharmacie, 34093 Montpellier Cedex 5, France. JMV 2959 doses were chosen based on locomotor activity studies done by Jerlhag's group (Jerlhag and Engel, 2011). Sodium pentobarbital was prepared by diluting a stock solution (Beuthanasia-D) with 0.9% saline to a final concentration of 100 mg/ml which was administered i.p. at a volume of 1 mg/ml. Ketamine solutions were prepared by mixing 80% ketamine (Ketaset: 80 mg/kg, Bioniche Pharma USA, Lake Forest, IL) and 20% xylazine (20 mg/kg).

Surgical Procedures

For the ICSS experiments (5 and 6), surgical implantation of stimulating electrodes was required. Surgical procedures follow those outlined in Wellman et al., 2008 (Wellman et

al., 2008a). Prior to surgery, each rat was injected (i.p.) with 0.4 mg/kg atropine sulfate (to minimize bronchial secretions), and then anesthetized using an injection (i.p.) of ketamine (Ketaset: 80 mg/kg) and xylazine (20 mg/kg). Each rat was mounted in a stereotaxic frame and the scalp incised using sterile technique. A 2% lidocaine jelly was applied to the incised edges of the scalp as pain relief to prevent scratching. The periosteum was mechanically retracted and skull bleeding was terminated using a styptic gel (Kwik-Stop, Gimborn Pet, Atlanta, GA). A bipolar stimulating electrode with 0.125-mm wire diameter (Plastics One, Roanoke, VA; No. 303/3) was implanted into the MFB at the level of the lateral hypothalamus. The incisor bar was set at -2.7 mm, and coordinates were 3.2 mm posterior to bregma, 1.7 mm lateral to the sagittal suture, and 8.3 mm ventral to the skull surface. Electrodes were affixed to the skull with three skull screws and dental acrylic (Lang Dental; Wheeling, IL). The lateral edges of the scalp incision were coated with a 0.1% gentamicin sulfate ointment (E. Fougera; Melville, NY) and the ends of the incision were closed using cyanoacrylate. Following surgery, each rat was injected (i.m.) with ampicillin (300,000 units). Butorphanol (0.05 mg/kg, SC: Dolorex) was used to alleviate post-surgical discomfort. A 7-day recovery period followed surgery, during which the rats were handled and weighed daily and had continuous access to water and food pellets in the home cage.

Histology Procedures

At the conclusion of the ICSS experiments, each rat was overdosed with sodium pentobarbital (100 mg/kg, i.p.), and perfused through the heart with 0.9% phosphate buffered saline followed by 10% formalin. Further fixation in 10% formalin/30% sucrose proceeded for at least 72 h prior to sectioning each brain. Alternate 80 um frozen sections were cover-

slipped for permanent storage. Coronal scans were compared to standard atlas plates (Paxinos and Watson, 2004) to verify electrode placements.

Apparatus

The assessment of locomotion was made in a set of 8 automated optical beam activity monitors (Model RXYZCM-16; Accuscan Instruments, Columbus, OH, USA). Each monitor was housed within a 40 X 40 X 30.5 cm acrylic cage. Activity monitors and cages were located in a sound-proof room with a 40 dB [SPL] white noise generator operating continuously. A multiplexor-analyzer monitored beam breaks from the optical beam activity monitors and tracked the simultaneous interruption of beams. The multiplexor-analyzer updated the animal's position in the acrylic cage every 10 ms using a 100% real-time conversion system. Computerized integration of the data obtained from the monitor afforded the recording of general activity using total distance (in cm) as the primary dependent measure.

For the ICSS experiments, the test chamber (Cambden Instruments) was constructed of Plexiglas and stainless steel with dimensions of 28x22x22 cm. Two levers were mounted on opposite sides of one wall 7 mm above the floor. Depression of the right lever was without consequence, while depression of the left lever resulted in the delivery of a 500-ms train of monophasic rectangular pulses with 1-ms pulse duration delivered from a Grass S88 stimulator (Grass Instruments, Quincy, MA) and a constant current stimulator (Model DS3; Digitimer, Hertfordshire, England) to the brain via a commutator and a flexible cable (Plastics One). All stimulation parameters were monitored on an oscilloscope (Model 645280; Jameco Electronics, Belmont, CA).

CHAPTER III

EXPERIMENT 1: EFFECT OF JMV 2959 ON COCAINE-INDUCED LOCOMOTOR SENSITIZATION*

Background

The literature suggests that ghrelin and GHS-Rs modulate reinforcement to addictive drugs that activate brain DA circuits (Jerlhag et al., 2006, Abizaid, 2009, Perello et al., 2010, Dickson et al., 2011). Consistent with this are studies mentioned previously that show systemic administration of ghrelin enhances COC-induced hyper locomotion (Wellman et al., 2005) and chronic daily injection of ghrelin in rats enhances locomotor sensitization to an acute injection of COC (Wellman et al., 2008b). Also, systemic and central administration of ghrelin can induce CPP (Jerlhag, 2008, Jerlhag et al., 2010), as well as enhance CPP induced by COC and by food (Davis et al., 2007, Egecioglu et al., 2010, Perello et al., 2010). Another way to assess the role of ghrelin in addictive drug effects involves inactivation of GHS-Rs.

Pharmacological antagonists of GHS-Rs were developed, at first because ghrelin is known to induce feeding, with the idea that inactivation of GHS-Rs could be used in the treatment of obesity. One of these antagonists is JMV 2959 which binds to GHS-Rs with low nanomolar affinity (Salome et al., 2009a). As would be expected of a GHS-R antagonist, s.c. administration of JMV 2959 dose-dependently blocked the feeding response induced by a synthetic ghrelin agonist hexarelin (Moulin et al., 2007a). JMV 2959 represents

* Part of this chapter is reprinted with permission from “Attenuation of cocaine-induced locomotor sensitization in rats sustaining genetic or pharmacologic antagonism of ghrelin receptors” Clifford, PS, Rodriguez, J, Schul, D, Hughes, S, Kniffin, T, Hart, N, Eitan, S, Brunel, L, Fehrentz, JA, Martinez, J, Wellman, PJ, 2011. *Addiction Biology* 17(6):956-63, Copyright 2011 by Wiley.

an important tool for the role of GHS-Rs in drug abuse. Pharmacological inactivation of GHS-Rs by JMV 2959 has been shown to attenuate or to ablate the acute locomotor and CPP properties of amphetamine, COC, ethanol, and most recently that of NIC (Jerlhag et al., 2009, Jerlhag et al., 2010, Jerlhag and Engel, 2011, Jerlhag et al., 2011). This experiment considered the impact of JMV 2959 on the development of locomotor sensitization induced by daily administration of COC in rats. Sensitization involves repeated exposures to a drug on a continuous basis. Repeated exposure produces an increase in the reaction to the drug on each of the successive days. An animal exposed to a single dose of COC will display an increase in locomotor response and the locomotor increase will be higher on day two after COC than they were on day one and so on. Locomotor sensitization is interpreted to reflect dynamic changes in the brain dopamine systems and these changes are assumed to be predictive of the ability of a drug to induce addiction. Put another way, locomotion is not addiction, but drugs that produce sensitization of locomotion are known to be addictive (Wise and Leeb, 1993). Drugs that have the capacity to block development of locomotor sensitization would presumably be useful for the prevention of COC addiction. Since COC acts in the NAC (Sellings et al., 2006), which contains GHS-Rs (Dickson et al., 2011), then antagonism of GHS-Rs would be expected to diminish the locomotor effects of COC. If antagonism of GHS-Rs diminishes the reinforcing effects of COC, ghrelin antagonists could prove useful in treatment of COC addiction.

Experiment Procedures

Subjects

The subjects of this experiment were 24 adult male Sprague-Dawley rats obtained from Harlan (Houston, Texas) weighing 250-275 g at the start of the experiment.

	Days: -5 to -4	Days: -3 to 0	Days: 1 - 7	
JMV2959 Coc Locomotion	Adaptation	Saline	Saline 20 Min Pre-Test	Saline
				Cocaine
			6 mg/kg JMV 2959 20 Min Pre-Test	Saline
				Cocaine

Figure 4. Behavioral Analysis of Experiment 1: Effect of JMV 2959 on COC-induced locomotor sensitization.

Behavioral Analysis

On two consecutive days, the rats were adapted to the locomotion chambers for 60 min per day. This adaptation period is essential because it serves to reduce baseline locomotion from the increased level seen when a rat encounters a novel environment (Miller et al., 1999). Over the next three days, the rats were injected with 0.9% saline (1 ml/kg) 5 min before being placed into the activity chamber. The rats were placed into the locomotion chambers for 15 min to habituate to the novel environment, removed and then injected with 0.9% saline and then placed back into the chambers for 45 min. During the 14 day COC exposure period, half of the rats in each injection condition were treated with either vehicle or 6 mg/kg JMV 2959 at 5 min before being placed into the locomotion chambers. After the 15 min baseline period, the rats were injected with either saline or 10 mg/kg COC hydrogen chloride and placed back into the locomotion chamber for 45 min. Therefore, there were 20 min between exposure to JMV 2959 and COC (see Figure 4). The reason for this is that it is known that JMV 2959 is rapidly absorbed and active in the brain during this lag period (Moulin et al., 2013). This pretreatment-treatment combination formed four test groups: vehicle-vehicle (n=5), vehicle-COC (n=5), JMV 2959-vehicle (n=7), and JMV 2959-COC (n=7).

Data Analysis

The overall design of the study was a split-plot (mixed) factorial design consisting of between-group factors of pretreatment status (vehicle versus 6 mg/kg JMV 2959) and COC exposure (vehicle versus 10 mg/kg COC) and a within-group factor of day. Because the treatment means and variances were proportional, the total distance traveled scores were

subjected to a log transformation (Kirk, 1982). Statistical significance was deemed to be $p < 0.05$ and the Bonferroni procedure was used to examine mean group differences.

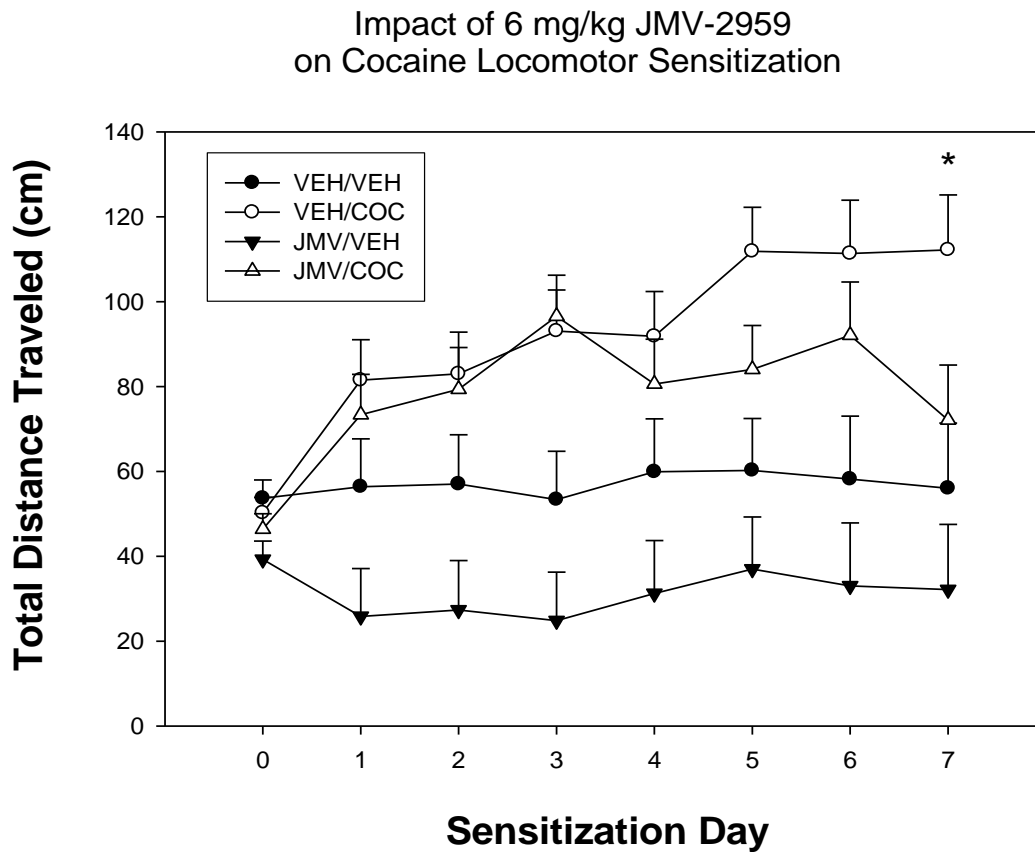


Figure 5. Impact of 6 mg/kg JMV 2959 on COC locomotor sensitization. Mean group total changes in total distance traveled scores (cm/45 min). On day 0, the rats were injected with vehicle at 10 min prior to the 15 min baseline period and then again with vehicle just prior to the 45 min test period. During days 1-7, the rats were injected with either vehicle (VEH) or 6 mg/kg JMV 2959 (JMV) at 10 min prior to the 15 min baseline period and then injected with either vehicle or 10 mg/kg COC (COC) just prior to the 45 min test period on days 1-7. The star (*) indicates a significant ($p < 0.05$) difference between the respective Veh and Coc groups. The lines above each symbol represent the S.E.M. Vehicle-vehicle (n=5), vehicle-COC (n=5), JMV 2959-vehicle (n=7), and JMV

Results

On the last day of the baseline procedure (Day 0 in Figure 5), there were no significant differences in 15 min locomotor scores ($p > 0.320$). Analyses of the 15 min scores, data not depicted, after administration of JMV 2959 (but before COC or vehicle) revealed a significant suppressive effect of JMV 2959 on baseline locomotion scores ($F(1,20) = 110.3, P < 0.0001$) as well as a significant interaction between the factors of day and JMV 2959 treatment ($F(7,140) = 12.9, P < 0.0001$). Analyses of the 45 min locomotor data on day 0 revealed no significant between group differences as a function of JMV 2959 treatment ($p = 0.334$), although there was a trend for the JMV-vehicle group to exhibit lower locomotion scores on Day 0 relative to the other groups. Additionally, the baseline scores revealed a significant inhibitory effect of JMV 2959 on locomotion during the first 15 min after administration. Accordingly, separate ANOVAs of the data were computed for the vehicle treatment and COC treatment conditions to compare the effect of JMV on locomotion. Considering the impact of JMV 2959 versus vehicle in rats treated with vehicle, ANOVA revealed no significant effect of JMV 2959 dose ($p = 0.058$), no effect of days ($p > 0.307$) and no interaction between JMV 2959 dose and days ($p = 0.9503$). The near significant trend for the JMV 2959 factor was attributed to the initial differences in these groups prior to the start of the JMV 2959 administration. A second analysis considered the impact of JMV 2959 in rats treated with 10 mg/kg COC. These analyses revealed no overall effect of JMV 2959 dose ($p = 0.497$), but revealed a significant effect of day ($F(6,72) = 12.5, p < 0.002$), as well as a significant interaction between JMV 2959 treatment and day ($F(6,72) = 4.228, p < 0.04$). The latter interaction reflected the fact that the JMV 2959-COC and vehicle-COC groups exhibited similar increases in locomotion during days 1-4, but the groups diverged during

days 5-7. In contrast, no such divergence was evident in the vehicle-vehicle and JMV 2959-vehicle groups. Indeed, the separation of the vehicle-vehicle and JMV 2959-vehicle groups were similar throughout the 7 days whereas the separation between the JMV 2959-COC and vehicle-COC groups was not evident until day 5 of the study.

Discussion

There is a growing body of evidence suggesting that modulation of CNS GHR activity can alter DA neuron circuits in rats and mice and in turn alter brain reinforcement function. These studies include the localization of GHS-Rs on DA neurons within the VTA (Guan et al., 1997, Naleid et al., 2005, Abizaid et al., 2006b, Diano et al., 2006, Abizaid, 2009) and the demonstration that systemic and intra-VTA administration of GHR can modulate DA release within the NAC (Jerlhag et al., 2006, Jerlhag et al., 2007, Quarta et al., 2009). Consistent with these studies, our laboratory has examined changes in the behavioral actions of COC in rats given supplemental doses of GHR. As expected, GHR administration facilitates acute COC hyper locomotion in rats (Wellman et al., 2005), induces a degree of behavioral sensitization to COC (Wellman et al., 2008b) and can facilitate CPP induced by low doses of COC (Davis et al., 2007).

The present study considered the development of locomotor sensitization induced by repeated administration of 10 mg/kg COC in rats for which GHR receptors were subject to pharmacological inactivation. With regard to GHS-R activity and baseline locomotion, the present results in which inactivation of GHS-Rs diminished baseline locomotion (see Figure 5) are consistent with other studies in which functional GHS-R activity is key to locomotion (Abizaid et al., 2006b, Jerlhag et al., 2006, Blum et al., 2009). The effect of JMV 2959 on

locomotion scores was significant for the first 15 min after treatment, but not for the next 45 min. The present experiment indicates pharmacological inactivation of GHS-Rs results in the attenuation of the development of COC locomotor sensitization. This effect was not evident during the initial hyperlocomotor effect of COC, but rather became evident after repeated COC exposures while the animals were in the process of developing locomotor sensitization.

CHAPTER IV

EXPERIMENT 2: EFFECT OF JMV 2959 ON NICOTINE-INDUCED LOCOMOTOR SENSITIZATION*

Background

The idea behind this experiment follows the same reasoning as in the first experiment, except NIC was used in place of COC. Previous studies from Jerlhag and Engel suggest that acute antagonism of GHS-Rs blocks the acute locomotor effects of NIC in mice (Jerlhag and Engel, 2011). Similar to COC, NIC has the capacity to induce CPP and repeated administration of NIC induces locomotor sensitization (Smith et al., 2010). Also, pharmacological antagonists of GHS-Rs might be useful in assisting in the cessation of smoking if it diminishes the rewarding properties of NIC, since it has already been shown that GHS-Rs antagonists have the capacity to decrease food intake. Central administration of JMV2959 suppresses ghrelin induced increases in body weight and fat mass, and blocks the ghrelin induced decreases in energy use (Salome et al., 2009b). This experiment considered the impact of JMV 2959 on the development of locomotor sensitization induced by daily administration of NIC in rats. Since NIC acts in the VTA where GHS-Rs are present, then antagonism of GHS-Rs would be expected to diminish the locomotor effects of NIC. If antagonism of GHS-Rs diminishes the reinforcing effects of NIC, ghrelin antagonists could prove useful in treatment of NIC addiction.

* Part of this chapter is reprinted with permission from “Pharmacologic antagonism of ghrelin receptors attenuates development of nicotine induced locomotor sensitization in rats” Wellman, PJ, Clifford, PS, Rodriguez, J, Hughes, S, Eitan, S, Brunel, L, Fehrentz, JA, Martinez, J, 2011. *Regulatory Peptides*, 172(1-3):77-80, Copyright 2011 by Elsevier.

Experimental Procedures

Subjects

The subjects of this experiment were 40 adult male Sprague-Dawley rats obtained from Harlan (Houston, Texas, USA) weighing 250-275 g at the start of the experiment.

	Days: -5 to -4	Days: -3 to 0	Days: 1 - 7	
JMV2959 Nic Locomotion	Adaptation	Saline	Saline 20 Min	Saline
			Pre-Test	Nicotine
			3 mg/kg JMV 2959 20 Min	Saline
			Pre-Test	Nicotine
			6 mg/kg JMV 2959 20 Min	Saline
			Pre-Test	Nicotine

Figure 6. Behavioral Analysis of Experiment 2: Effect of JMV 2959 on NIC-induced locomotor sensitization.

Behavioral Analysis

On two consecutive days, the rats adapted to the locomotion chambers for 60 min per day. On the next three days, the rats were injected (i.p.) with 0.9% saline (1 ml/kg) 5 min before being placed into the activity chamber. The last day of the vehicle injection trials served as day 0 for baseline. The rats were placed into the locomotion chambers for 15 min, removed and then injected with 0.9% saline and then placed back into the chambers for an additional 45 min. During the 7 day NIC exposure period, a third of the rats in each NIC injection condition were treated (i.p.) with vehicle (0), 3 or 6 mg/kg JMV 2959 at 5 min before being placed into the locomotion chambers. After the 15 min baseline period, the rats were injected (s.c.) with either saline or 0.4 mg/kg NIC HT then placed back into the locomotion chamber for 45 min (see Figure 6). This pretreatment-treatment combination formed six test groups: vehicle-vehicle (n=6), vehicle-NIC (n=6), 3 mg/kg JMV 2959-vehicle (n=8), 3 mg/kg JMV 2959-NIC (n=8), 6 mg/kg JMV 2959-vehicle (n=6), and 6 mg/kg JMV 2959-NIC (n=6).

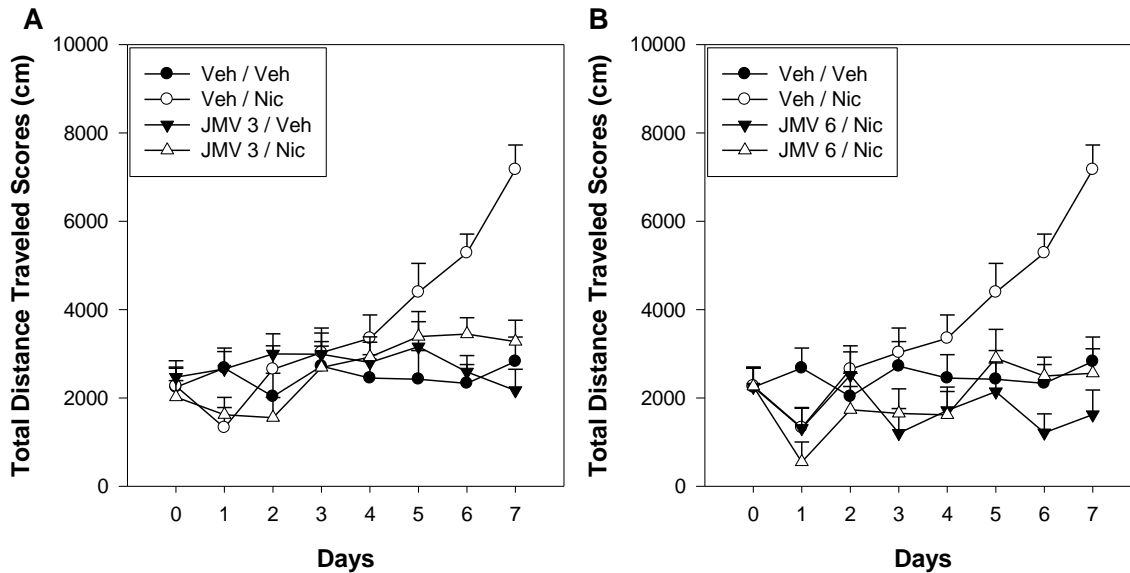


Figure 7. Impact of 3 and 6 mg/kg JMV 2959 on NIC locomotor sensitization.

Mean group total changes in total distance traveled scores (cm/45 min). On day 0, the rats were injected with Veh at -5 min prior to the 15 min baseline period and then again with Veh just prior to the 45 min test period. During days 1-7, the rats were injected with either Veh, 3 mg/kg JMV 2959 (JMV 3: panel A) or 6 mg/kg JMV 2959 (JMV 6: panel B) at -5 min prior to the 15 min baseline period and then injected with either vehicle or 0.4 mg/kg NIC (Nic) just prior to the 45 min test period on days 1-7. The lines above and below each symbol represent the SEM. The star (*) indicates a significant ($p < 0.05$) difference between the respective Veh and Nic groups.

Vehicle-vehicle (n=6), vehicle-NIC (n=6), 3 mg/kg JMV 2959-vehicle (n=8), 3 mg/kg JMV 2959-NIC (n=8), 6 mg/kg JMV 2959-vehicle (n=6), and 6 mg/kg JMV 2959-NIC (n=6).

Results

On the last day of the baseline procedure (Day 0 in panels A and B of Figure 7), there were no significant effects of NIC dose ($F(1,34) = 0.17, P < 0.685$) or of JMV 2959 dose ($F(1,34) = 0.001, P < 0.999$), and there was no significant interaction among these factors ($F(2,34) = 0.24, P < 0.788$) on baseline 45 min locomotion scores. A split-plot ANOVA of

the changes in locomotion induced by 0, 3 or 6 mg/kg JMV 2959 across the treatment days in vehicle treated rats (data depicted in Panels A and B of Figure 7) revealed no significant effect of days ($F(7,119) = 0.556, P < 0.790$), or of JMV 2959 dose ($F(2,17) = 1.448, P < 0.254$), nor was there a significant interaction between days and JMV 2959 dose ($F(14,119) = 1.055, P < 0.405$). Although there was a trend for 6 mg/kg JMV 2959 to reduce locomotion on some days, these differences were not significant nor was there an effect of 3 mg/kg JMV 2959 on locomotion in rats treated with vehicle.

An overall split-plot ANOVA was computed to determine the impact of NIC (0 vs. 0.4 mg/kg) and of JMV 2959 (0, 3 or 6 mg/kg) on locomotion across the 8 days of this experiment. These analyses revealed a significant overall effect of day ($F(1,34) = 40.33, p < 0.0001$) as well as interactions between NIC dose and day ($F(1,34) = 48.24, p < 0.0001$), JMV 2959 dose and day ($F(2,34) = 9.89, p < 0.0001$), and a triple interaction between NIC dose, JMV 2959 dose and day ($F(2,34) = 4.79, p < 0.015$).

Results with NIC and JMV 2959 are depicted in the three panels of Figure 7. In panel A, administration of 0.4 mg/kg NIC in vehicle pretreated rats initially suppressed locomotion on treatment day 1 and over the next 6 days, this treatment resulted in significant sensitization of locomotion to levels nearly three times that noted in vehicle treated rats. Post hoc contrasts revealed significant differences ($p < 0.05$) between the Veh-Veh group and the Veh-Nic group on days 5, 6, and 7. In panels A and B, rats pretreated with either 3 or 6 mg/kg JMV 2959 and then treated with NIC showed an initial (but non-significant) suppression of locomotion lasting 1-2 days, but failed to exhibit a subsequent significant enhancement of locomotion as was induced by 0.4 mg/kg NIC in vehicle pretreated rats. In

panels A and B, no contrasts between Veh and Nic treatments were significant for any dose of JMV 2959.

Discussion

The present study considered the development of locomotor sensitization induced by repeated administration of 0.4 mg/kg NIC in rats for which GHR receptors were antagonized by pretreatment with the GHS-R antagonist JMV 2959 (Moulin et al., 2007a, Salome et al., 2009b). Rats that were pre-treated with JMV 2959 showed significant attenuation of the development of hyperlocomotion to daily injections of 0.4 mg/kg NIC. However, when JMV 2959 was administered by itself, the 6 mg/kg dose slightly reduced locomotion but the 3 mg/kg dose did not (see Figure 7). This outcome parallels a recent study done in Long-Evans rats showing behavior disruptions when these rats were given 6 mg/kg JMV 2959, but no behavioral disruptions were seen in rats given lower doses such as 1, 2 or 3 mg/kg JMV 2959 (Landgren et al., 2012). In the present study, both 3 and 6 mg/kg JMV 2959 produced similar attenuation of the development of NIC-induced hyper locomotion. What this suggests is that the attenuation of locomotor sensitization to NIC is not wholly due to JMV 2959 disrupting baseline locomotion in and of itself. The blunted development of NIC locomotor sensitization reported herein is similar in direction (but not magnitude) to what is seen in experiment 1 in which the same 6 mg/kg of JMV 2959 was noted to attenuate the sensitization induced by daily injection of 10 mg/kg COC in rats (Clifford et al., in press). Taken together, these results strongly implicate functional GHS-R activity as required for the induction of locomotor sensitization by psychostimulants.

Acute administration of JMV 2959 has been shown to reduce locomotion, block the induction of CPP and blunt the ability of NIC to increase locomotion and DA release in the

accumbens in mice (Jerlhag and Engel, 2011). These effects are likely due to activation by NIC of nicotinic cholinergic afferents projecting to the VTA, which in turn activate DA overflow within the NAC (Jerlhag et al., 2006). This study looks at chronic pharmacological inactivation of GHS-Rs and the ability of GHS-R antagonists to significantly diminish the locomotor sensitization induced by NIC in rats, the process of NIC sensitization is unknown.

Since GHS-Rs are critically involved in the induction of eating (Tschop et al., 2000, Abizaid, 2009, Egecioglu et al., 2010), antagonism of GHS-Rs has been a key focus of appetite suppressant drug development. Pharmacological antagonism of GHS-Rs can diminish baseline feeding and attenuate the rewarding action of food (Egecioglu et al., 2010, Perello et al., 2010). This experiment shows that antagonism of GHS-R function and its ability to reduce the development of NIC sensitization may be useful in the treatment for NIC addiction. Such an outcome may suggest that GHS-R drug antagonists may have multiple avenues for the treatment of NIC addiction. The first way it can combat NIC addiction is by diminishing the rewarding action of NIC. Another way to combat NIC addiction might be to prevent the weight gain often noted following cessation of smoking. Oftentimes this weight gain is an important enough barrier to prevent people from quitting smoking (Pomerleau and Saules, 2007). Additionally, a distinct haplotype of the GHS-R is associated with smoking risk in low-level female consumers of alcohol, though how it affects ghrelin signaling is yet unknown (Landgren et al., 2010).

In contrast to the impact of inhibition of GHR signaling on COC behavioral function, our earlier laboratory studies showed that administration of GHR facilitates COC-induced hyper locomotion and COC-induced CPP (Wellman et al., 2005, Davis et al., 2007, Wellman et al., 2008b). Repeated administration of feeding-relevant doses of ghrelin (5, 10 nMol)

induces a cross sensitization to COC, which augments COC stimulated locomotor responses (Wellman et al., 2008b). In other words, rats repeatedly exposed to ghrelin will respond to their first exposure to COC with higher locomotor responses than do rats that were exposed with vehicle. This study needs to be replicated with regards to NIC. This could be indicative of how ghrelin is known to reorganize inputs in the reward pathway particularly in the VTA to result in neural activation (sensitization) (Abizaid et al., 2006b). This effect may also be related to an up-regulation of D1 receptors such that ghrelin can amplify DA signaling (Jiang et al., 2006). Though, this is in opposition to our experiments showing that antagonism of GHS-Rs diminishes the development of sensitization to NIC and to COC. A more general role for GHS-Rs in brain reinforcement is also indicated by recent studies in which pharmacological inactivation of GHS-Rs attenuates the CPP induced by ethanol (Jerlhag et al., 2009) and in which genetic ablation of GHS-Rs attenuates the CPP induced by ingestion of high-fat foods (Perello et al., 2010). GHS-Rs also play a key role in the consumption of sweet tasting food and drink in rats and humans (Landgren et al., 2011, Skibicka et al., 2011a, Skibicka et al., 2011b). These converging outcomes strongly support the view that GHR receptors modulate reinforcement/reward function.

CHAPTER V

EXPERIMENT 3: EFFECT OF GHRELIN RECEPTOR KNOCKOUT ON COCAINE-INDUCED LOCOMOTOR SENSITIZATION*

Background

The rationale for these next two experiments is the same as in the first two experiments with a change to the genetic knockout strategy for inactivation of ghrelin signaling. Ghrelin inactivation strategies include immunosuppression (Lu et al., 2009), RNA silencing (Shrestha et al., 2009) GHS-R antagonists (Halem et al., 2004, Abizaid et al., 2006b), and gene knockout strategies, primarily in mice (Abizaid et al., 2006b, Sun et al., 2008). Few studies have been done in GHS-R knockout rats. The next two experiments should provide a convergence of the pharmacological antagonism and genetic knockout methods in rats. This experiment considered the impact of genetic ablation of GHS-Rs on the development of locomotor sensitization induced by daily administration of COC in rats.

COC seems to produce its locomotor effects through activation in the NAC (Sellings et al., 2006) where GHS-Rs are present (Dickson et al., 2010). If development of those receptors is prevented through genetic knockout, the expected result would be that development of locomotor sensitization due to COC would be diminished in rats sustaining GHS-R knockout.

* Part of this chapter is reprinted with permission from “Attenuation of cocaine-induced locomotor sensitization in rats sustaining genetic or pharmacologic antagonism of ghrelin receptors” Clifford, PS, Rodriguez, J, Schul, D, Hughes, S, Kniffin, T, Hart, N, Eitan, S, Brunel, L, Fehrentz, JA, Martinez, J, Wellman, PJ, 2011. *Addiction Biology* 17(6):956-63, Copyright 2011 by Wiley.

Experimental Procedures

Subjects

The subjects of this experiment were 24 adult male FHH rats described above (see General Methods), obtained from (PhysGen Program in Genomic Applications) weighing 275-300 g at the start of the experiment.

	Animals Obtained	Days -5 Through -4	Days -3 to 0	Days 1 - 14
GHS-R ^(-/-) Coc Locomotion	WT	Adaptation	Saline	Saline
				Cocaine
	GHS-R ^(-/-)	Adaptation	Saline	Saline
				Cocaine

Figure 8. Behavioral Analysis of Experiment 3: Effect of ghrelin receptor knockout on COC- induced locomotor sensitization.

Behavioral Analysis

Animals were separated into WT and GHS-R^(-/-) groups based on genotype. On two consecutive days, the rats were adapted to the locomotion chambers for 45 min per day. On the next three days, the rats were placed in the chamber for 15 min, removed and injected with 0.9% saline and then placed back into the chambers for 45 min. Test animals within each group were then randomly assigned to receive intraperitoneal (i.p) injections of either vehicle (0.9% saline) or 10.0 mg/kg COC hydrogen chloride for 14 successive days, thus forming four test groups: WT/vehicle (n=4), WT/COC (n=7), GHS-R^(-/-)/vehicle (n=5) and GHS-R^(-/-)/COC (n=8). Few rats were available for the study and so a decision was made to increase the group size for the COC treatment group. During sensitization testing, animals were placed in their respective test chambers for a 15 min baseline-recording period prior to receiving either a vehicle or COC injection. Rats were then placed back in the chamber immediately following injection, at which time recording continued for another 45 min (see Figure 8).

Data Analysis

The overall design of the study was a split-plot (mixed) factorial design consisting of between-group factors of GHR receptor status (WT versus GHS-R^(-/-)) and COC exposure (vehicle versus 10 mg/kg COC) and a within-group factor of day (blocks 1-7 were formed using averages of 2 days total distance data). Because the treatment means and variances were proportional, the total distance traveled scores were subjected to a log transformation (Kirk, 1982). Statistical significance was deemed to be $p < 0.05$ and the Bonferroni procedure was used to examine mean group differences.

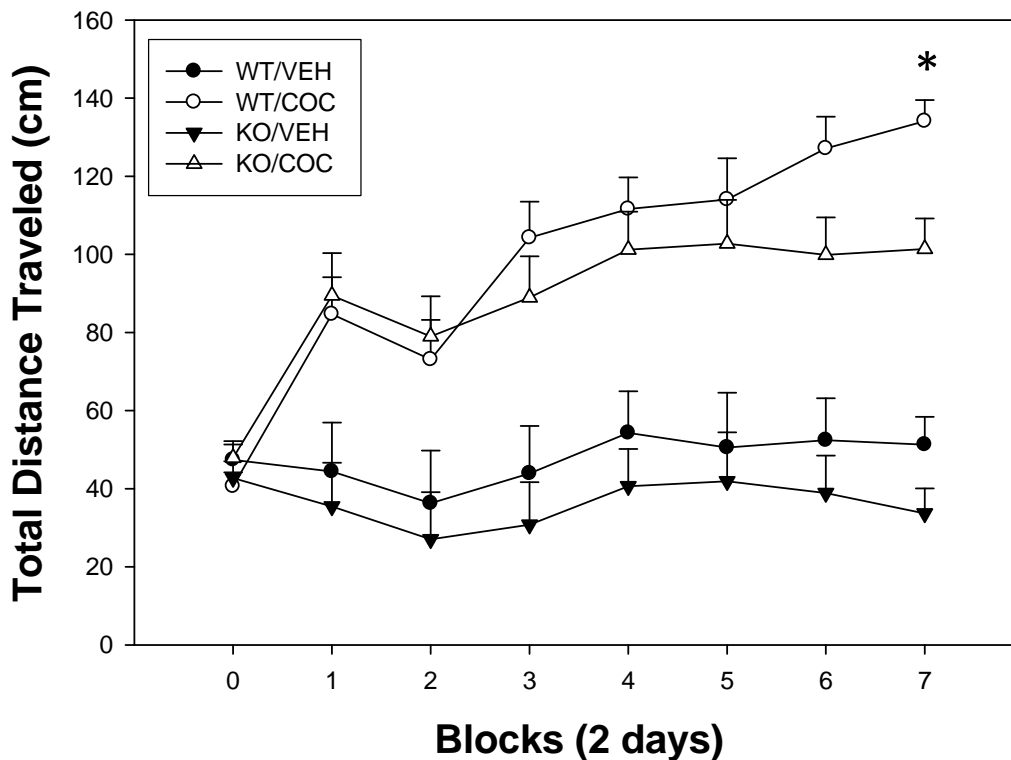


Figure 9. Impact of GHS-R KO on cocaine locomotor sensitization.

Mean group total changes in total distance traveled scores (cm/45 min) for WT and GHS-R^(-/-) (KO) rats injected with vehicle on Day 0 and then with either vehicle (VEH) or 10 mg/kg COC just prior to the 45 min test period on days 1-14. The lines above each symbol represent the S.E.M. The star (*) indicates a significant ($p < 0.05$) difference between the respective Veh and COC groups. WT/vehicle (n=4), WT/cocaine (n=7), GHS-R^(-/-)/vehicle (n=5) and GHS-R^(-/-)/cocaine (n=8).

Results

A two-way ANOVA revealed no significant ($p > \text{at least } 0.156$) effect of GHR receptor status, COC exposure nor an interaction among these factors on locomotion scores

after vehicle (block 0 in Figure 9). Although there were no initial differences between the WT and GHS-R^(-/-) groups treated with vehicle, these groups diverged over the 7 blocks such that by the last block, the GHS-R^(-/-) rats treated with vehicle showed significantly less locomotion than did the WT rats treated with vehicle. A two-way ANOVA for the data on block 7 was computed using change in locomotion from respective baselines. This ANOVA revealed a significant effect of COC exposure ($F(1,20) = 129.1, p < 0.0001$), a significant effect of GHR gene status ($F(1,20) = 15.6, p < 0.0001$), and a significant interaction between COC exposure and GHR gene status ($F(1,20) = 4.1, p < 0.05$). The latter interaction reflected the fact that the difference in locomotion scores between the WT and GHS-R^(-/-) rats during the last block relative to baseline was significantly larger in the COC exposure condition than in the vehicle treatment condition.

Discussion

In an earlier study, it was noted that rats sustaining mutation of the GHS-R resulting in a functional ablation of that receptor do not overeat when given systemic ghrelin injections (Figure 10) and show diminished locomotor sensitization to daily injections of COC, data from (Clifford et al., 2012). Similarly, Abizaid and colleagues noted that ghrelin knockout mice exhibit diminished locomotor sensitization to COC (Abizaid et al., 2011). Collectively, these studies suggest that GHS-Rs play a prominent role in the development of behavioral sensitization to psychostimulants. Moreover, the present results extend earlier studies in which GHS-Rs were noted to be required for the induction of hyper locomotion to COC, to amphetamine, and to ethanol (Jerlhag, 2008, Abizaid, 2009, Jerlhag et al., 2009, Jerlhag et al., 2010, Jerlhag and Engel, 2011).

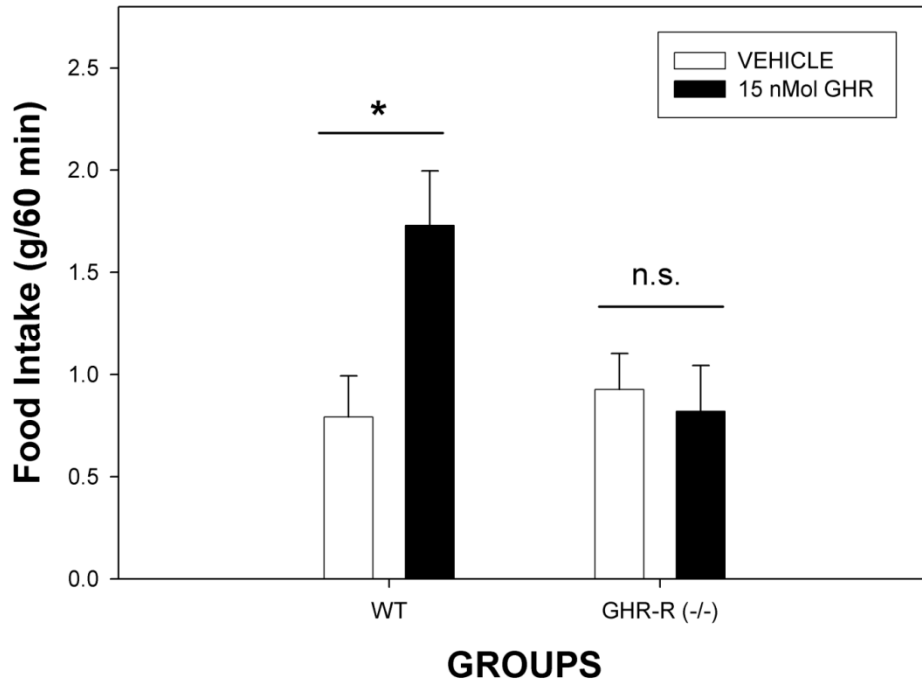


Figure 10. Changes in food intake in WT and GHS-R^(-/-) rats after ghrelin. Mean food intakes in grams over a one hour period following injection of vehicle or 15 nMol ghrelin in WT and GHS-R^(-/-) rats. The lines above each bar represent the S.E.M. WT rats responded to ghrelin with a significant increase in food intake compared to vehicle whereas the GHS-R^(-/-) rats did not (Data unpublished). The star (*) indicates a significant ($p < 0.05$) difference between the respective groups.

The key outcome of this experiment was that GHS-R^(-/-) rats exhibited attenuated development of COC sensitization over a 14 day exposure period (see Figure 9). This outcome indicates that activation of GHR receptors plays a modest role in the development of COC sensitization. The blunted development of COC locomotor sensitization reported herein parallels a recent study by Jerlhag (Jerlhag et al., 2010) in which administration of a GHS-R antagonist attenuated the acute hyper locomotion induced by the psychostimulants COC as well as amphetamine; reduced the increase in accumbens DA produced by COC and

most importantly attenuated COC-induced CPP. In contrast to the impact of inhibition of GHR signaling on COC behavioral function, our earlier laboratory studies showed that administration of GHR facilitates COC-induced hyper locomotion and COC-induced CPP (Wellman et al., 2005, Davis et al., 2007, Wellman et al., 2008b).

A more general role for GHRs in brain reinforcement is also indicated by recent studies in which pharmacological inactivation of GHS-Rs attenuates the CPP induced by ethanol (Jerlhag et al., 2009) whereas genetic ablation of GHRs attenuates the CPP induced by ingestion of a high-fat diet (Perello et al., 2010). The converging outcomes of this experiment and experiment 1 strongly support the view that GHR receptors modulate DA function and drug-induced reinforcement/reward.

CHAPTER VI

EXPERIMENT 4: EFFECT OF GHRELIN RECEPTOR KNOCKOUT ON NICOTINE-INDUCED LOCOMOTOR SENSITIZATION

Background

The background for this experiment is identical to that of experiment 2 for the rationale behind using NIC, and is identical to that of experiment 3 for the rationale for using GHS-R knockout. This experiment considered the impact of genetic ablation of GHS-Rs on the development of locomotor sensitization induced by daily administration of NIC in rats.

Just as in the second experiment, NIC seems to produce its effects through the VTA where a large number of GHS-Rs are present (Abizaid, 2009). If activation of those receptors is prevented through genetic knockout, the expected result would be that locomotor sensitization due to NIC would be diminished in rats sustaining GHS-R knockout and to an even greater degree than seen in COC-induced locomotor sensitization.

Experimental Procedures

Subjects

The subjects of this experiment were 27 adult male FHH rats described above, obtained from (PhysGen Program in Genomic Applications) weighing 275-300 g at the start of the experiment.

	Animals Obtained	Days -5 Through -4	Days -3 to 0	Days 1 - 10
GHS-R(-/-) Nic Locomotion	WT	Adaptation	Saline	Saline
				Nicotine
	GHS-R(-/-)	Adaptation	Saline	Saline
				Nicotine

Figure 11. Behavioral Analysis of Experiment 4: Effect of ghrelin receptor knockout on NIC- induced locomotor sensitization.

Behavioral Analysis

Animals were separated into WT and GHS-R^(-/-) groups based on genotype. On two consecutive days, the rats were adapted to the locomotion chambers for 45 min per day. On the next three days, the rats were placed in the chamber for 15 min, removed and injected with 0.9% saline and then placed back into the chambers for 45 min. Test animals within each group were then randomly assigned to receive i.p injections of either vehicle (0.9%

saline) or subcutaneous (s.c.) injections of 0.4 mg/kg NIC hydrogen tartrate for 10 successive days, thus forming four test groups: WT/vehicle (n=4), WT/NIC (n=8), GHS-R^(-/-)/vehicle (n=4) and GHS-R^(-/-)/NIC (n=11). There were once again a limited number of animals available so a decision was made to increase the group size of the NIC treatment group. During sensitization testing, animals were placed in their respective test chambers for a 15 min baseline-recording period prior to receiving either a vehicle or NIC injection. Rats were then placed back in the chamber immediately following injection, at which time the room lights again were turned off and recording continued for another 45 min (see Figure 11).

Results

A two-way ANOVA revealed no significant ($p >$ at least 0.245) effect of GHR receptor status, NIC exposure nor an interaction among these factors on locomotion scores after vehicle (day 0 in Figure 12). A two-way ANOVA for the data on day 9 revealed a significant effect of NIC exposure ($F(1,23) = 19.75, p < 0.05$), GHR gene status ($F(1,23) = 7.71, p < 0.05$), and interaction between NIC exposure and GHR gene status ($F(1,23) = 5.86, p < 0.05$). These interactions reflected that the difference in locomotion scores between the WT and the GHS-R^(-/-) rats during those days relative to baseline was significantly larger in rats receiving NIC than in rats receiving vehicle. ANOVA for day 10 showed similar significant effects of NIC exposure ($F(1,23) = 21.70, p < 0.05$), GHR gene status ($F(1,23) = 9.48, p < 0.05$), but not an interaction between NIC exposure and GHR gene status ($F(1,23) = 3.73, p < 0.07$).

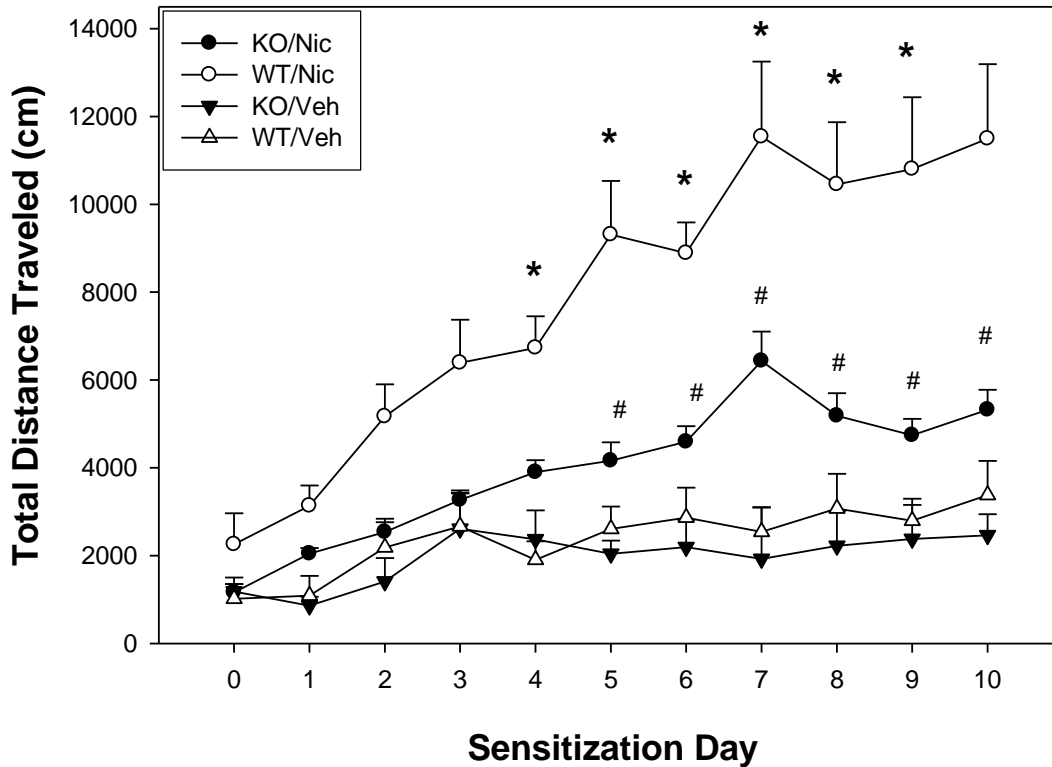


Figure 12. Impact of GHS-R KO on NIC locomotor sensitization. The results show that WT-Vehicle rats exhibit stable locomotion scores across the 10-day testing period. In contrast, WT-NIC rats were sensitized to daily NIC treatment over the testing period. GHS-R^(-/-) rats and WT rats treated with vehicle did not show any differences in baseline locomotion. Importantly, GHS-R^(-/-) rats treated with NIC (0.4 mg/kg) show less sensitization over the test period in contrast to those rats in the WT-NIC condition. WT/vehicle (n=4), WT/NIC (n=8), GHS-R^(-/-)/vehicle (n=4) and GHS-R^(-/-)/NIC (n=11). The star (*) indicates a significant (p < 0.05) difference between WT/NIC and KO/NIC. The (#) indicates a significant (p < 0.05) difference between KO/NIC and KO/Veh.

Discussion

The key outcome of this experiment was that GHS-R^(-/-) rats exhibited attenuated development of NIC sensitization over a 10 day exposure period. This outcome indicates that activation of GHR receptors plays a role in the development of NIC sensitization. The blunted development of NIC locomotor sensitization here supports experiment 2, which showed pharmacological antagonism of GHS-Rs attenuated the development of NIC locomotor sensitization. The converging outcomes of this experiment and experiment 3 strongly support the view that GHS-Rs modulate DA function and drug-induced reinforcement/reward particularly for NIC.

CHAPTER VII

EXPERIMENT 5: EFFECT OF JMV 2959 ON NICOTINE-ENHANCED INTRACRANIAL SELF-STIMULATION*

Background

The next two experiments should provide a convergence of the pharmacological antagonism and genetic knockout methods in rats. Examining locomotor sensitization gives us a good idea about the stimulatory effects of psycho-stimulants. However, it doesn't provide as much information into the rewarding effects of psycho-stimulants that a drug self-administration method gives us. Few studies have been done relating ghrelin to drug self-administration. Serum levels of ghrelin have been shown to rise preceding periods of reinstatement for COC (Tessari et al., 2007); these increases significantly predict COC reinstatement. Preliminary studies from this lab have suggested that JMV 2959 suppresses both COC, as well as NIC self-administration (Rodriguez, unpublished data). There are other ways to look at reward other than looking at how drugs are self-administered. It has been demonstrated that electrical stimulation of the brain can result in rewarding effects (Olds and Milner, 1954). Many drugs of abuse facilitate electrical stimulation of the MFB, and this electrical stimulation is sensitive to reinforcement (i.e. increased by hunger, decreased by DA antagonists (Wise, 1996). There are many methods for investigating ICSS, but rate-frequency, described below, is likely the most useful.

* Part of this chapter is reprinted with permission from "Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fMRI and intracranial self-stimulation" Wellman, PJ, Clifford, PS, Rodriguez, JA, Hughes, S, Di Francesco, C, Melotto, S, Tessari, M, Corsi, M, Bifone, A, Gozzi, A, 2011. *Addiction Biology*, 17(5):908-19, Copyright 2011 by Wiley.

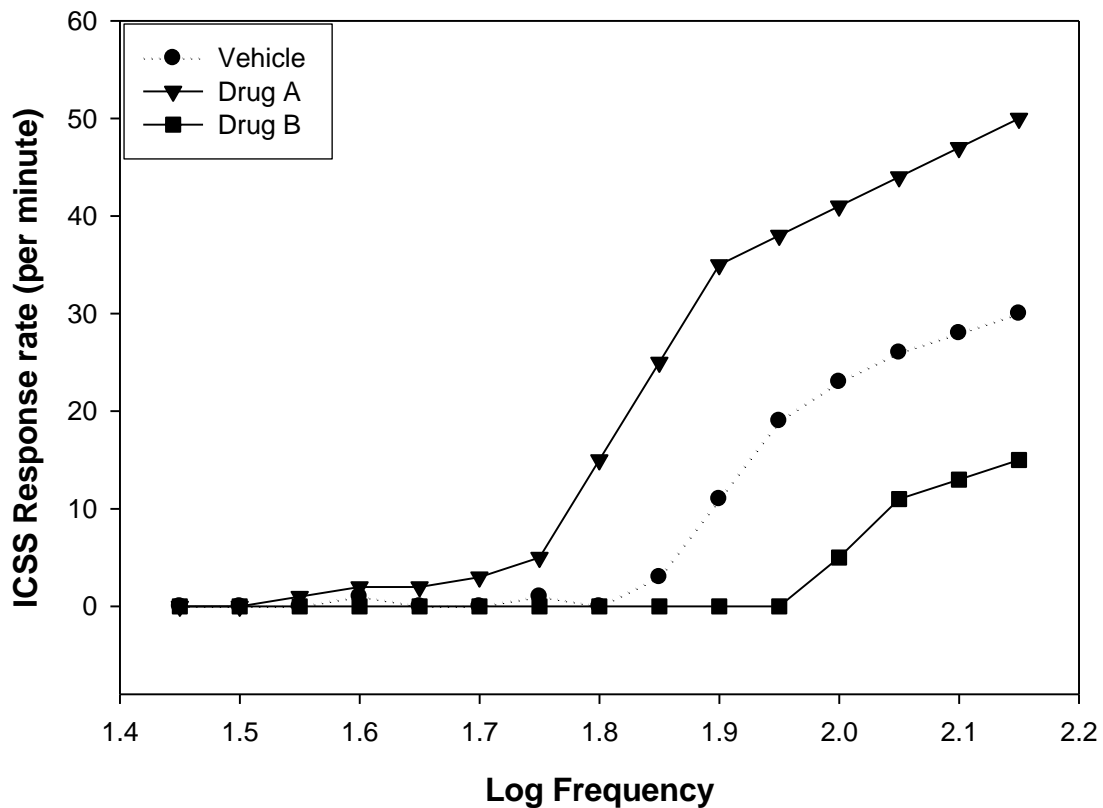


Figure 13. Theoretical ICSS Construct

The construct shown in Figure 13 details what the results should look like in the ICSS paradigm. The middle curve, with circles as data points, is an example of the vehicle baseline responding. The upper curve, with triangles as data points, shows what is expected to happen if a drug is administered that facilitates the rewarding effects of the electrical stimulation. This is a leftward shift in the rate-frequency curve where the 50% response rate is reached at a lower frequency than is required in the vehicle condition and the 100% response rate exceeds the rate seen in the vehicle group. The lower curve, with squares as

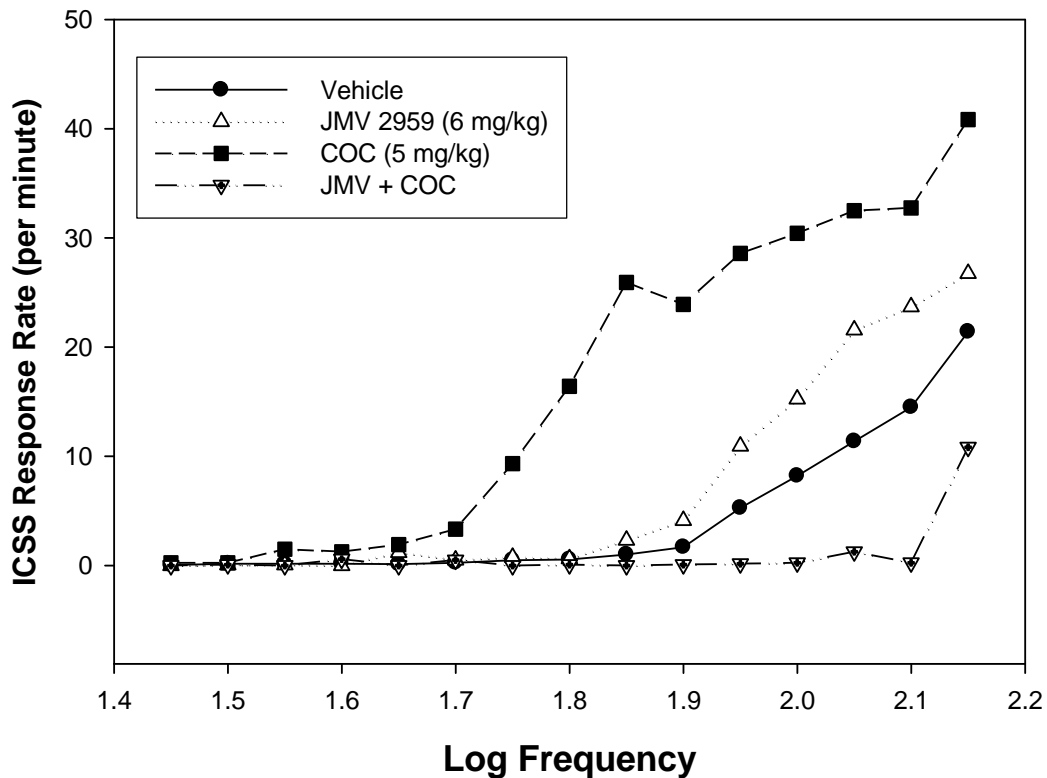


Figure 14. Impact of JMV 2959 and cocaine on rate-frequency responding in ICSS. The results show rate frequency curves for rats treated with vehicle, cocaine (5 mg/kg), JMV 2959 (6 mg/kg) or a combined pretreatment of JMV 2959 and cocaine. Cocaine both increased the 100% response rate and induced a slight left-ward shift of the curve (consistent with augmented reinforcement). In contrast, pretreatment with JMV 2959 reversed the left-ward shift and generally suppressed ICSS responding. JMV alone did not significantly alter responding. Data unpublished.

data points, shows what should happen if a drug is administered that attenuates the rewarding effects of the electrical stimulation. This is called a rightward shift in the rate-frequency curve the 50% response rate of the vehicle group is never reached and the 100% response rate falls far short of the rate seen in the vehicle group. In an ICSS task like the one performed here in the next two experiments, rats press a lever for a pleasurable electrical stimulation of their brain at varying frequencies. Infusion of ghrelin causes a rightward shift in responding (unpublished data, Kniffin thesis). In a recent experiment, rats were trained and tested in the same paradigm with COC being the drug tested instead of NIC. COC both increased the 100% response rate and induced a slight left-ward shift of the curve (consistent with augmented reinforcement). In contrast, pretreatment with JMV 2959 reversed the left-ward shift and generally suppressed ICSS responding. JMV alone did not significantly alter responding (see Figure 14).

This experiment considered the impact of NIC and JMV 2959 on ICSS responding, both separately and in combination. Since NIC and COC facilitate electrical stimulation in the MFB, the expected result would be an effect similar to what was seen with COC (Figure 14).

Experimental Procedures

Subjects

The subjects of this experiment were 5 adult male Sprague-Dawley rats obtained from Harlan (Houston, Texas) weighing 250-275 g at the start of the experiment.

	Surgery	Recovery	Shaping	Day: 1		Day: 2 & 3
JMV2959 Nic ICSS	Electrodes Implanted	5 Days Recovery	Rats Shaped	Saline 20 Min Pre- Test	Saline	Intertrials
				Saline 20 Min Pre- Test	Nicotine	
				6 mg/kg JMV 2959 20 Min Pre-Test	Nicotine	

Figure 15. Behavioral Analysis of Experiment 5: Effect of JMV 2959 on NIC-enhanced intracranial self-stimulation

Behavioral Analysis

After recovery from surgery, each rat was shaped to lever-press for rewarding brain stimulation on a fixed ratio-1 schedule. During shaping, current intensity was systematically increased until a minimum rewarding current (a current sufficient to elicit lever responding) is reached (typically between 50-150 μ A). Once the lever-pressing behavior is acquired,

animals were run through 75 min baseline trials consisting of five separate 15-min passes. During each 15-min pass, the intensity was kept constant while the frequency of stimulation was lowered each min from 141 Hz to 28 Hz (decreasing in 0.05 log units). During testing, each rat was run multiple trials on separate days and was injected (i.p.) with either vehicle, NIC (0.25 mg/kg), or a combination of JMV 2959 (3 mg/kg) and NIC (0.25 mg/kg). JMV 2959 was injected 20 min before the beginning of each trial and vehicle and NIC were both injected 5 min before the beginning of each trial. Two days of vehicle trials were interposed between each drug trial (see Figure 15). The number of lever-presses per min was recorded for each rat throughout each 75 min trial.

Data Analysis

Data from the first pass was discarded for each daily test (Carlezon and Chartoff, 2007). For each rat and session, the total number of responses, rate-frequency curve, maximal response rate (100% response rate), 50% response rate, and threshold (frequency which produced 50% response rate) was computed using the responses from the last 4 daily passes. Maximal response rate, 50% response rate, and threshold were analyzed.

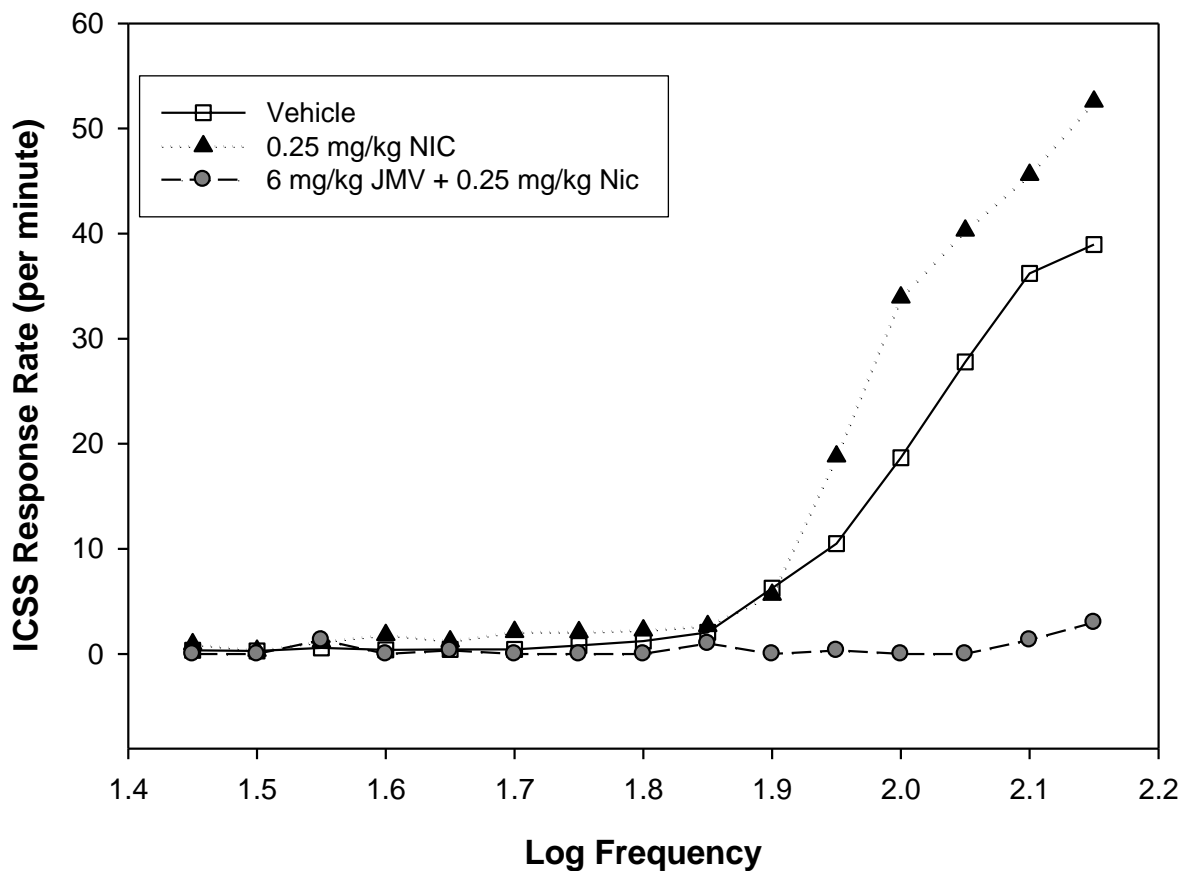


Figure 16. Impact of JMV 2959 and NIC on rate-frequency responding in ICSS. The results show rate frequency curves for rats treated with vehicle, NIC (0.25 mg/kg) or a combined pretreatment of JMV 2959 and NIC. NIC both increased the 100% response rate and induced a slight left-ward shift of the curve (consistent with augmented reinforcement). In contrast, pretreatment with JMV 2959 reversed the left-ward shift and generally suppressed ICSS responding. N=5.

Results

Relative to vehicle condition, NIC slightly increased the response rate and shifted the curve to the left (see Figure 16). Pretreatment with JMV 2959 suppressed responding in NIC treated rats relative to the vehicle condition. Analysis of the 100% response rate did not show a significant increase in responding in NIC treated rats compared to vehicle ($t(8) = -1.358, p$

= 0.21) but showed a significant decrease in responding in rats given JMV 2959 and NIC compared to vehicle rats ($t(8) = 5.79, p < 0.001$). Analysis of the 50% response rate showed a significant increase in responding following NIC administration compared to vehicle ($t(8) = -3.104, p < 0.05$). Analysis of the JMV 2959 with NIC condition was not possible at the 50% rate because there was no responding and therefore there was no variability. All electrode placements were within the lateral hypothalamus between 1.9 and 3.72 mm posterior to bregma.

Discussion

In experiment 5, NIC increased the 50% response rate, slightly increased the 100% response rate and induced a slight left-ward shift of the curve (consistent with augmented reinforcement; Figure 16). This is consistent with an earlier experiment in which COC increased the 50% and 100% response rate and induced a slight left-ward shift of the curve in the same paradigm. In contrast, pretreatment with JMV 2959 reversed the left-ward shift in both the NIC and COC paradigms and generally suppressed ICSS responding. The experiment presented here looks at ICSS at low intensity, but it is as yet unclear whether increasing the current intensity would be sufficient to return ICSS responding to baseline or above in rats pretreated with JMV 2959 and challenged with NIC.

CHAPTER VIII

EXPERIMENT 6: EFFECT OF GHRELIN RECEPTOR KNOCKOUT ON INTRACRANIAL SELF-STIMULATION*

Background

There are two main methods of inactivating the ghrelin system. One approach is through the use of GHS-R antagonists, such as JMV 2959, which has been discussed earlier. The other primary method of inactivating the ghrelin system is to genetically knockout the development of GHS-Rs. The rationale for looking at ICSS is described in section 1 of chapter VII. This experiment considered the impact of genetic ablation of GHS-Rs on ICSS responding.

Experimental Procedures

Subjects

The subjects of this experiment were 11 adult male FHH rats described above, obtained from (PhysGen Program in Genomic Applications) weighing 275-300 g at the start of the experiment. Subjects were housed according to the same procedures listed in chapter II.

* Part of this chapter is reprinted with permission from “Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fMRI and intracranial self-stimulation” Wellman, PJ, Clifford, PS, Rodriguez, JA, Hughes, S, Di Francesco, C, Melotto, S, Tessari, M, Corsi, M, Bifone, A, Gozzi, A, 2011. *Addiction Biology*, 17(5):908-19, Copyright 2011 by Wiley.

GHS-R(-/-) ICSS	Animals Obtained	Surgery	Recovery	Shaping	Day: 1
	WT	Electrodes Implanted	5 Days Recovery	Rats Shaped	Testing Day
	GHS-R(-/-)				

Figure 17. Behavioral Analysis of Experiment 6: Effect of ghrelin receptor knockout on intracranial self-stimulation

Behavioral Analysis

Animals were separated into WT (n=5) and GHS-R^(-/-) (n=6) groups based on genotype. After recovery from surgery, each rat was shaped to lever-press for rewarding brain stimulation on a fixed ratio-1 schedule. During shaping, current intensity was systematically increased until a minimum rewarding current (a current sufficient to elicit lever responding) is reached (typically between 50-150 μ A). Once the lever-pressing behavior is acquired, animals were run through 75 min baseline trials consisting of five separate 15-min passes (see Figure 17). During each 15-min pass, the intensity was kept

constant while the frequency of stimulation was lowered each min from 141 Hz to 28 Hz (decreasing in 0.05 log units).

Data Analysis

Data from the first pass was discarded for each daily test (Carlezon and Chartoff, 2007). For each rat and session, the total number of responses, rate-frequency curve, maximal response rate (100% response rate), 50% response rate, and threshold (frequency which produced 50% response rate) were computed using the responses from the last 4 daily passes. Maximal response rate, 50% response rate, and threshold were analyzed.

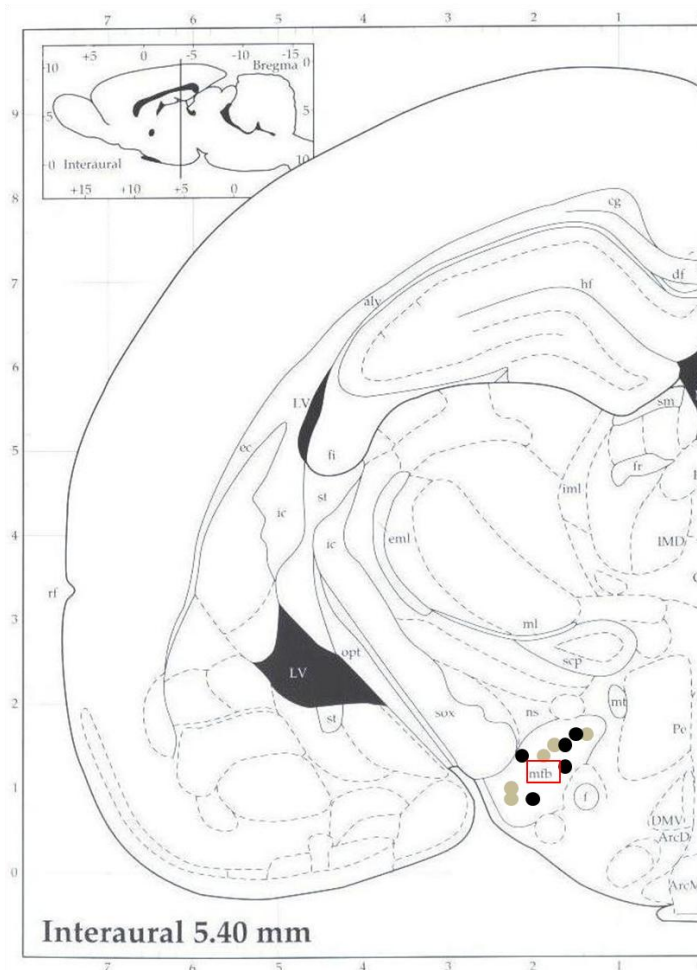


Figure 18. Typical ICSS Electrode Placements. All electrode placements were within the lateral hypothalamus between 1.9 and 3.72 mm posterior to bregma (plates derived from Paxinos and Watson (2006)).

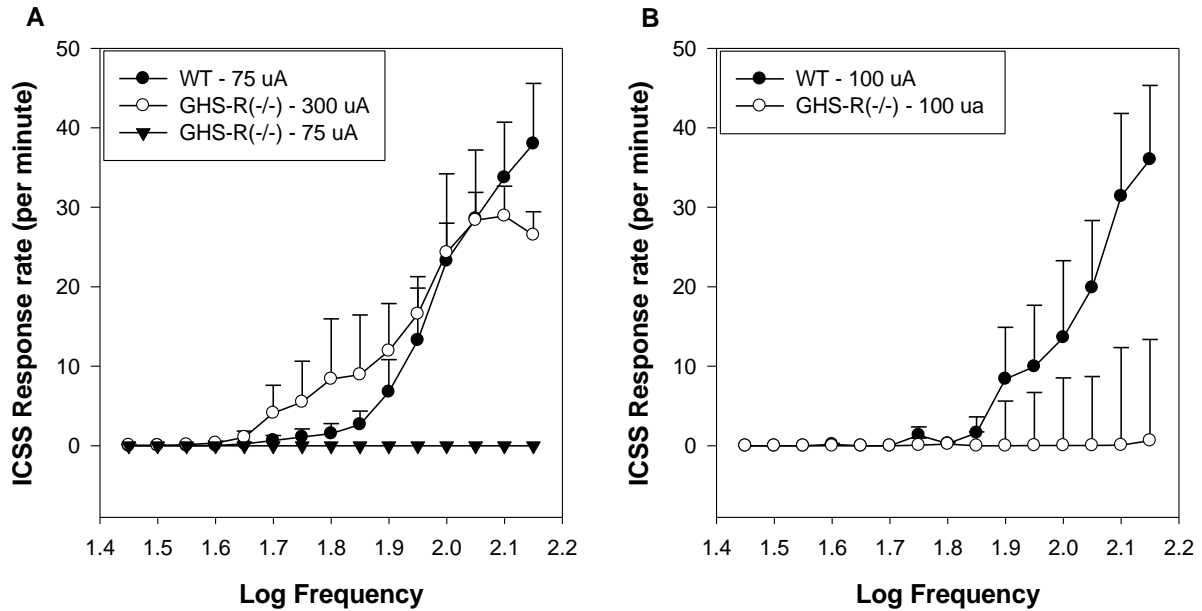


Figure 19. Impact of GHS-R KO on rate-frequency responding in ICSS. Rate frequency curves for GHS-R^(-/-) rats (N=6) and WT rats (N=5) as a function of stimulation intensity. Panel A: WT rats tested at 75 μ A and GHS-R^(-/-) rats tested at 300 μ A show similar rate frequency curves. WT- and GHS-R^(-/-) rats tested at 300 μ A were not significantly different in terms of total responses or 50% threshold responses. When GHS-R^(-/-) rats were tested at 75 μ A, the rats did not lever press for ICSS (and thus the error bars were zero). Panel B: GHS-R^(-/-) rats tested at 100 μ A fail to respond for ICSS, whereas WT rats show a typical rate frequency curve. Calculation of threshold response was not possible for GHS-R^(-/-) rats tested at 100 μ A. WT (n=5) and GHS-R(-/-) (n=6).

Results

In this experiment, GHS-R^(-/-) rats were used to investigate whether GHR directly influences the functional activity of brain reinforcement circuits. This issue was addressed by examining the reinforcing action of ICSS in wild-type (WT) and GHS-R^(-/-) rats.

Rats normally rapidly acquire ICSS responding at stimulation intensities of 75-100 μ A for placements within the lateral hypothalamus (see Figure 18) (Burkey and Nation,

1994). In the present study, GHS-R^(-/-) rats failed to acquire ICSS at stimulation intensities of 75-100 μ A during our initial shaping procedure, but did acquire responding when intensity was increased to 300 μ A. Figure 19 depicts the rate-frequency curves for GHS-R^(-/-) rats (tested at 300 μ A) versus WT rats tested at 75 μ A. These curves were mostly overlapping suggesting that GHS-R^(-/-) rats show the same general function (i.e. similar total responses at the higher stimulation frequencies and a systematic decrease in response rate as stimulation frequency is decreased) but at different stimulation intensities. At the end of rate frequency testing, the two groups were retested at a common stimulation intensity of 100 μ A. As can be seen in Figure 19, the GHS-R^(-/-) rats failed to respond for ICSS to any frequency, when tested at 100 μ A. All electrode placements were within the lateral hypothalamus between 1.9 and 3.72 mm posterior to bregma (see Figure 18).

Discussion

In this experiment, GHS-R^(-/-) rats failed to acquire ICSS at stimulation intensities (\sim 75 μ A) sufficient to motivate ICSS in WT rats. These GHS-R^(-/-) rats only acquired the ICSS response when the current intensity was increased to more than 300 μ A. After the intensity was raised to sufficient intensities, the overall rate-frequency curve of the null rats was similar to the curve seen in WT rats (see Figure 19). Following rate-frequency testing, the GHS-R^(-/-) rats were shifted back to an intensity of 100 μ A, at which point, ICSS responding ceased at all frequencies. This suggests that the GHS-Rs may not be required for ICSS responding, but does strongly suggest a facilitative role for these receptors in ICSS. One explanation for this outcome is that the stimulating electrodes employed in this experiment were located within the MFB, at the level of the lateral hypothalamus, and that ICSS of this

site activated DA fibers coursing from the VTA to the NAcc. GHS-Rs have a marked constitutive action (Petersen et al., 2009) which would be expected to provide activation of brain neurons in the absence of ghrelin. Ablation of this facilitative action on VTA DA activity would in turn be expected to diminish ICSS function. Non-dopaminergic factors may be involved since there are relatively few DA fibers within the MFB (Yeomans, 1989, Wise, 2002, 2004) and by that ICSS of the lateral hypothalamus induces Fos formation in dopaminergic and non-dopaminergic brain sites, such as the substantia nigra, the raphe nuclei and the locus coeruleus (Ishida et al., 2001) as well as the VTA. Another explanation for this outcome could be that the GHS-R^(-/-) rats had developed brain structures differently, or failed to develop them fully, due to a lifelong absence of GHS-Rs. It is, as yet, unclear what the scope of differences that occur in the absence of GHS-Rs compared to WT rats. It could also be that once the intensities were ramped up so high, the stimulating pulses could be affecting a larger area thus stimulating surrounding tissues.

The ENU-based mutation of the GHS-R (resulting in a truncated GHS-R protein sequence) is a relatively novel null model of GHS-R function. As mentioned before, GHS-R^(-/-) rats fail to overeat in response to systemic injection of acylated GHR, whereas WT rats significantly increase their food intakes (Clifford et al., 2012). As noted in the previous experiments, there was an attenuated development of locomotion to daily injection of 10 mg/kg (i.p.) COC in both GHS-R^(-/-) rats, as well as rats that were pretreated with JMV 2959, a pharmacological antagonist of the GHS-R (Moulin et al., 2007a, Salome et al., 2009b). These results suggest a key role for GHS-Rs in the development of locomotor sensitization, which is consistent with a role for GHS-Rs in reinforcement processes. A more general role for GHS-Rs in brain reinforcement is also indicated by recent studies in which

pharmacological inactivation of GHS-Rs attenuates the hyperlocomotor effects, the release of accumbens DA and the CPP induced by alcohol (Jerlhag et al., 2009), NIC (Jerlhag and Engel, 2011), and amphetamine and COC (Jerlhag et al., 2010). With regard to ethanol, there is evidence that blockade of GHS-Rs diminishes alcohol consumption and alcohol self-administration (Landgren et al., 2012) and that genetic variation of the GHS-R can be associated with human alcohol overconsumption (Landgren et al., 2009, Landgren et al., 2010). Finally, GHS-Rs modulate the reinforcing effects that accrue to consumption of food. Systemic GHR administration increases food consumption and food reward (Disse et al., 2010, Dickson et al., 2011, Skibicka et al., 2011b) while blockade of GHS-Rs can suppress consumption and associated preference for palatable foods including sweets and foods high in fat (Egecioglu et al., 2010, Perello et al., 2010, Skibicka et al., 2011b). Collectively, these studies strongly support the importance of GHS-R signaling for reinforcement; whether that reinforcement is associated with eating, drug ingestion or ICSS.

CHAPTER IX

GENERAL DISCUSSION AND CONCLUSION*

Experiment 1 examined the development of locomotor sensitization induced by repeated administration of 10 mg/kg COC in rats for which GHR receptors were subject to pharmacological inactivation via a 6 mg/kg dose of JMV 2959. These results in which inactivation of GHS-Rs diminished the development of COC sensitized locomotion (see Figure 5) are consistent with other studies in which functional GHS-R activity is key to COC stimulated locomotion (Abizaid et al., 2006b, Jerlhag et al., 2006, Blum et al., 2009). This result indicates that pharmacological inactivation of GHS-Rs attenuates the development of COC locomotor sensitization. This effect was not evident during acute administration of COC, but rather was revealed after chronic COC exposures while the animals were in the process of developing sensitization. One problem with this experiment is the lack of variation in the dosages. The 10 mg/kg dose of COC was chosen because in past studies it produced the highest level of responding without causing any aversion, but other doses of JMV could be used to further the power of this experiment.

Experiment 2 considered the development of locomotor sensitization induced by repeated administration of 0.4 mg/kg NIC in rats for which GHR receptors were antagonized via JMV 2959 (Moulin et al., 2007a, Salome et al., 2009b). JMV 2959 significantly attenuated the development of hyperlocomotion to daily injections of 0.4 mg/kg NIC. However, JMV 2959 administered by itself was not sufficient to reduce locomotion at 3

* Part of this chapter is reprinted with permission from “Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fMRI and intracranial self-stimulation” Wellman, PJ, Clifford, PS, Rodriguez, JA, Hughes, S, Di Francesco, C, Melotto, S, Tessari, M, Corsi, M, Bifone, A, Gozzi, A, 2011. *Addiction Biology*, 17(5):908-19, Copyright 2011 by Wiley.

mg/kg, and the 6 mg/kg dose reduced locomotion only a modest amount (see Figure 7). As mentioned before, this outcome is similar to another recent study done in Long-Evans rats showing that 6 mg/kg JMV 2959 produced behavior disruptions, but not at lower doses such as 1, 2 or 3 mg/kg JMV 2959 (Landgren et al., 2012). In experiment 2, both 3 and 6 mg/kg JMV 2959 produced similar attenuation of the development of NIC-induced hyperlocomotion. These results suggest that the attenuation of locomotor sensitization to NIC is not simply JMV 2959 disrupting baseline locomotion by itself. The blunted development of NIC locomotor sensitization reported here is similar in direction to what is seen in experiment 1 (compare Figures 5 and 7) in which the same 6 mg/kg JMV 2959 dose was noted to attenuate the sensitization induced by daily injection of 10 mg/kg COC in rats (Clifford et al., in press).

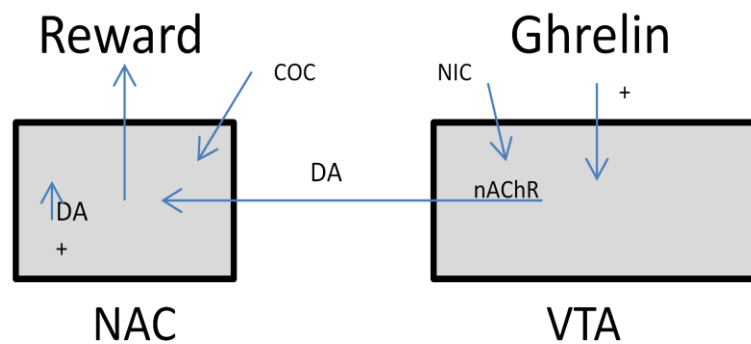


Figure 20. Simplified GHS-R functionality circuit

Interestingly, the effect that JMV 2959 has on NIC induced locomotor sensitization is more pronounced than its effect on COC induced locomotor sensitization. This might be due to the fact that COC and NIC produce sensitization via different pathways. COC acts through the NAC via blockade of reuptake to produce locomotor effects whereas NIC acts to increase DA fiber firing rate within the VTA, which in turn induces DA activation in the VTA (see Figure 20). There is a heavy concentration of GHS-Rs within the VTA but these are sparse within the NAC. While it may be difficult to infer functional activity from relative receptor number, one possible explanation for this pattern of results is that GHS-Rs have a greater linkage to NIC-induced locomotor activation and thus JMV 2959 has a greater impact than it does for COC-induced locomotion (which lies within the NAC with relatively few GHS-Rs).

In experiment 3, rats sustaining GHS-R knockout exhibited attenuated development of COC sensitization. This indicates that activation of GHS-Rs plays at least a modest role in the development of COC sensitization. The blunted development of COC locomotor sensitization reported herein parallels the results of experiment 1 where JMV 2959 diminished COC induced locomotor sensitization (see Figure 9). This outcome is similar to the outcome of a recent study by Jerlhag (Jerlhag et al., 2010) in which administration of a GHS-R antagonist attenuated the acute hyper locomotion induced by COC. Considering that both pharmacological antagonism and genetic ablation of GHS-Rs both diminish COC induced locomotor sensitization, experiments 1 and 3 together provide a convergence of methods to inactivate ghrelin signaling.

In experiment 4, rats sustaining GHS-R knockout exhibited attenuated development of NIC sensitization (see Figure 12). This suggests that activation of GHS-Rs plays a role in

the development of NIC sensitization. This outcome is similar to what is shown in experiment 2 where JMV 2959 attenuated NIC induced locomotor sensitization (compare Figures 9 and 12). Since experiments 2 and 4 demonstrate that pharmacological antagonism and genetic ablation of GHS-Rs attenuates the development of NIC-induced locomotor sensitization, once again there is a convergence of methods to inactivate ghrelin signaling and of outcomes. This convergence makes the data that much more compelling. Comparing the outcomes of experiments 1 and 2 showed a greater impact of JMV 2959 on NIC induced locomotor sensitization than COC induced locomotor sensitization. This difference is also seen in experiments 3 and 4 showing that genetic ablation of GHS-Rs has a larger impact on NIC induced locomotor sensitization compared to COC. This is also likely due to NIC and COC acting through different pathways. The loss of GHS-Rs in the VTA is greater than the loss would be in the NAC which should result in a larger effect, which is shown here.

In experiment 5, NIC both increased the 100% self-stimulation response rate and induced a slight left-ward shift of the curve (consistent with augmented reinforcement) (see Figure 16). This parallels an earlier experiment in which COC was used in NIC's place and COC also increased the 100% response rate and induced a slight left-ward shift of the curve (see Figure 14). In contrast, pretreatment with JMV 2959 reversed the left-ward shift in both the NIC and COC paradigms and generally suppressed ICSS responding. In this instance, the effect was more pronounced in the COC condition compared to NIC (compare Figures 14 and 16). This is likely due to electrodes being located within the MFB, and that ICSS of this site activated DA fibers coursing from the VTA to the NAC.

In experiment 6, rats sustaining GHS-R knockout were unable to acquire ICSS at stimulation intensities ($\sim 75 \mu\text{A}$) that are sufficient to motivate ICSS in WT rats (see Figure

19). It was not until the current intensity was ramped up to more than 300 μA that these GHS-R^(-/-) rats were able to acquire ICSS responding. After the intensity was raised high enough to elicit responding, the overall rate-frequency curve of the null rats was similar to the curve seen in WT rats. Following rate-frequency testing, the GHS-R^(-/-) rats were returned to an intensity of 100 μA , at which point, ICSS responding ceased at all frequencies. This suggests that the GHS-Rs may not be required for ICSS responding, but rather acts in a facilitative role for these receptors in ICSS. Abizaid noted that activation of GHS-Rs resulted in more excitability of DA fibers (due to greater glutamate activity) within the VTA (and less inhibitory GABA inputs) (Abizaid et al., 2006b). This could be because the stimulating electrodes employed in this experiment were located within the MFB, at the level of the lateral hypothalamus, and that ICSS of this site activates DA fibers coursing from the VTA to the NAC. Not all of these reinforcement fibers involve DA (Wellman et al., 2012) and it may be possible that with higher current intensities, non-DA fibers (which are not influenced by ghrelin or GHS-Rs) are recruited, resulting in ICSS. Based on the results of this experiment, it would be of great interest to see if raising the current intensity of rats pretreated with JMV 2959 and challenged with NIC or COC would be sufficient to rescue their ICSS responding.

Together, these experiments provide a strong basis for the therapeutic effects of GHS-R antagonism in relation to drug reward. The important outcome to take away from these series of experiments is the consistent suppression of NIC and COC induced locomotion and reinforcement by JMV 2959. The GHS-R knockout studies serve to support and extend the studies involving JMV 2959. It is important to note that JMV 2959 has a consistent profile of shutting down the effects of NIC and COC. Considering the results of experiments 1 and

2, JMV 2959 could prove useful in the treatment of addiction, at least in the prevention of reinstatement of COC or NIC. From the data presented here, the impact of GHS-R antagonism seems to be greater with NIC rather than COC. This is quite fortuitous because the scope of NIC addiction is far greater than the scope of COC addiction. The use of JMV 2959 in the prevention of acquisition of drug abuse is obviously impractical, but it could be of use as a counter measure to addiction (i.e., to assist in stopping drug-taking and to prevent relapse after drug cessation). One of the added benefits of JMV 2959 is that it also acts on feeding behavior to reduce food intake. The cessation of smoking has been repeatedly linked to an instance of weight gain. Many female smokers have reported an unwillingness to quit smoking if it meant gaining even 5 pounds (Pomerleau, 1986). JMV 2959 could possibly aid in the cessation of smoking while simultaneously reducing food intake and preventing the weight gain association with quitting.

In the future, it would be important to examine other GHS-R antagonists to see if they result in the same attenuations of the development of drug sensitization. Also, GHS-R antagonists that do not cross the blood brain barrier would be useful to look at to block the leftward shifts seen in the ICSS experiments to see if the effect is central or peripheral. Also, as touched upon earlier, it would be nice to look at the effects of vagal GHS-Rs.

Immunization against GHS-Rs might be an avenue of study in the future. There is already work being done with drug immunizations against COC use (Koob et al., 2011), but GHS-R inactivation could prove more useful. Another future study should look at NIC in the periphery and JMV 2959 in the VTA to see if it blocks the locomotor effects and the DA change in the NAC. It would also be important to determine if there are gender differences with regard to modulation of ghrelin signaling and drug reinforcement.

The first follow up that needs to be addressed, however, is to look at NIC self administration in GHS-R^(-/-) rats and rats pretreated with JMV 2959 or other GHS-R antagonists. ICSS is a proxy for drug addiction, but the gold standard is drug self-administration. There is some preliminary unpublished data from our laboratory showing that pretreatment with JMV 2959 inhibits i.v. self-administration of 0.5 mg/kg/infusion of COC and to 0.03 mg/kg/infusion of NIC (Rodriguez, 2012, unpublished data). This needs to be extended further with the same conditions presented here.

REFERENCES

- Abizaid A (2009) Ghrelin and dopamine: new insights on the peripheral regulation of appetite. *J Neuroendocrinol* 21:787-793.
- Abizaid A, Gao Q, Horvath TL (2006a) Thoughts for food: brain mechanisms and peripheral energy balance. *Neuron* 51:691-702.
- Abizaid A, Horvath TL (2008) Brain circuits regulating energy homeostasis. *Regul Pept* 149:3-10.
- Abizaid A, Liu ZW, Andrews ZB, Shanabrough M, Borok E, Elsworth JD, Roth RH, Sleeman MW, Picciotto MR, Tschop MH, Gao XB, Horvath TL (2006b) Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *J Clin Invest* 116:3229-3239.
- Abizaid A, Mineur YS, Roth RH, Elsworth JD, Sleeman MW, Picciotto MR, Horvath TL (2011) Reduced locomotor responses to cocaine in ghrelin-deficient mice. *Neuroscience* 192:500-506.
- Al Massadi O, Tschop MH, Tong J (2011) Ghrelin acylation and metabolic control. *Peptides* 32:2301-2308.
- Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) *Molecular Biology of the Cell*. New York: Garland Science.
- Andrews ZB, Erion D, Beiler R, Liu ZW, Abizaid A, Zigman J, Elsworth JD, Savitt JM, DiMarchi R, Tschop M, Roth RH, Gao XB, Horvath TL (2009) Ghrelin promotes and protects nigrostriatal dopamine function via a UCP2-dependent mitochondrial mechanism. *J Neurosci* 29:14057-14065.

- Asakawa A, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino MA, Kasuga M (2003) Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut* 52:947-952.
- Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D, Muller EE (2003) Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. *Regul Pept* 111:161-167.
- Banks WA, Burney BO, Robinson SM (2008) Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood-brain barrier. *Peptides* 29:2061-2065.
- Banks WA, Tschop M, Robinson SM, Heiman ML (2002) Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 302:822-827.
- Becskei C, Bilik KU, Klusmann S, Jarosch F, Lutz TA, Riediger T (2008) The anti-ghrelin Spiegelmer NOX-B11-3 blocks ghrelin- but not fasting-induced neuronal activation in the hypothalamic arcuate nucleus. *J Neuroendocrinol* 20:85-92.
- Bell SM, Stewart RB, Thompson SC, Meisch RA (1997) Food-deprivation increases cocaine-induced conditioned place preference and locomotor activity in rats. *Psychopharmacology (Berl)* 131:1-8.
- Bennett PA, Thomas GB, Howard AD, Feighner SD, van der Ploeg LH, Smith RG, Robinson IC (1997) Hypothalamic growth hormone secretagogue-receptor (GHS-R) expression is regulated by growth hormone in the rat. *Endocrinology* 138:4552-4557.
- Bevins RA, Palmatier MI (2003) Nicotine-conditioned locomotor sensitization in rats: assessment of the US-preexposure effect. *Behav Brain Res* 143:65-74.

- Blum ID, Patterson Z, Khazall R, Lamont EW, Sleeman MW, Horvath TL, Abizaid A (2009) Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. *Neuroscience* 164:351-359.
- Brown LM, Benoit SC, Woods SC, Clegg DJ (2007) Intraventricular (i3vt) ghrelin increases food intake in fatty Zucker rats. *Peptides* 28:612-616.
- Brunetti L, Recinella L, Orlando G, Michelotto B, Di Nisio C, Vacca M (2002) Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. *Eur J Pharmacol* 454:189-192.
- Burkey RT, Nation JR (1994) Brain stimulation reward following chronic lead exposure in rats. *Behav Neurosci* 108:532-536.
- Carlezon WA, Jr., Chartoff EH (2007) Intracranial self-stimulation (ICSS) in rodents to study the neurobiology of motivation. *Nat Protoc* 2:2987-2995.
- Carlini VP, Ghersi M, Schioth HB, de Barioglio SR (2010) Ghrelin and memory: differential effects on acquisition and retrieval. *Peptides* 31:1190-1193.
- Carlini VP, Monzon ME, Varas MM, Cragolini AB, Schioth HB, Scimonelli TN, de Barioglio SR (2002) Ghrelin increases anxiety-like behavior and memory retention in rats. *Biochem Biophys Res Commun* 299:739-743.
- Carroll ME, Lynch WJ, Roth ME, Morgan AD, Cosgrove KP (2004) Sex and estrogen influence drug abuse. *Trends Pharmacol Sci* 25:273-279.
- Chuang JC, Zigman JM (2010) Ghrelin's Roles in Stress, Mood, and Anxiety Regulation. *Int J Pept* 2010.
- Clifford PS, Rodriguez J, Schul D, Hughes S, Kniffin T, Hart N, Eitan S, Brunel L, Fehrentz JA, Martinez J, Wellman PJ (2012) Attenuation of cocaine-induced locomotor

- sensitization in rats sustaining genetic or pharmacologic antagonism of ghrelin receptors. *Addiction Biology* 17:956-963.
- Clifford S, Zeckler RA, Buckman S, Thompson J, Hart N, Wellman PJ, Smith RG (2011) Impact of food restriction and cocaine on locomotion in ghrelin- and ghrelin-receptor knockout mice. *Addict Biol* 16:386-392.
- Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649-661.
- Davidson TL, Kanoski SE, Tracy AL, Walls EK, Clegg D, Benoit SC (2005) The interoceptive cue properties of ghrelin generalize to cues produced by food deprivation. *Peptides* 26:1602-1610.
- Davis KW, Wellman PJ, Clifford PS (2007) Augmented cocaine conditioned place preference in rats pretreated with systemic ghrelin. *Regul Pept* 140:148-152.
- de Lartigue G, Dimaline R, Varro A, Dockray GJ (2007) Cocaine- and amphetamine-regulated transcript: stimulation of expression in rat vagal afferent neurons by cholecystokinin and suppression by ghrelin. *J Neurosci* 27:2876-2882.
- Depoortere I (2009) Targeting the ghrelin receptor to regulate food intake. *Regul Pept* 156:13-23.
- Diano S, Farr SA, Benoit SC, McNay EC, da Silva I, Horvath B, Gaskin FS, Nonaka N, Jaeger LB, Banks WA, Morley JE, Pinto S, Sherwin RS, Xu L, Yamada KA, Sleeman

- MW, Tschop MH, Horvath TL (2006) Ghrelin controls hippocampal spine synapse density and memory performance. *Nat Neurosci* 9:381-388.
- Dickson SL, Egecioglu E, Landgren S, Skibicka KP, Engel JA, Jerlhag E (2011) The role of the central ghrelin system in reward from food and chemical drugs. *Mol Cell Endocrinol* 340:80-87.
- Dickson SL, Hrabovszky E, Hansson C, Jerlhag E, Alvarez-Crespo M, Skibicka KP, Molnar CS, Liposits Z, Engel JA, Egecioglu E (2010) Blockade of central nicotine acetylcholine receptor signaling attenuate ghrelin-induced food intake in rodents. *Neuroscience* 171:1180-1186.
- Disse E, Bussier AL, Veyrat-Durebex C, Deblon N, Pfluger PT, Tschop MH, Laville M, Rohner-Jeanrenaud F (2010) Peripheral ghrelin enhances sweet taste food consumption and preference, regardless of its caloric content. *Physiol Behav* 101:277-281.
- Drazen DL, Vahl TP, D'Alessio DA, Seeley RJ, Woods SC (2006) Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology* 147:23-30.
- Egecioglu E, Jerlhag E, Salome N, Skibicka KP, Haage D, Bohlooly YM, Andersson D, Bjursell M, Perrissoud D, Engel JA, Dickson SL (2010) Ghrelin increases intake of rewarding food in rodents. *Addict Biol* 15:304-311.
- Figlewicz DP, Sipols AJ (2010) Energy regulatory signals and food reward. *Pharmacol Biochem Behav* 97:15-24.
- Fulton S (2010) Appetite and reward. *Front Neuroendocrinol* 31:85-103.

- Garko MG (2011) Overweight and obesity epidemic in America – Part X: The fat future of America. In: Health and Wellbeing Monthly.
- Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* 48:23-29.
- Halem HA, Taylor JE, Dong JZ, Shen Y, Datta R, Abizaid A, Diano S, Horvath T, Zizzari P, Bluet-Pajot MT, Epelbaum J, Culler MD (2004) Novel analogs of ghrelin: physiological and clinical implications. *Eur J Endocrinol* 151 Suppl 1:S71-75.
- Halem HA, Taylor JE, Dong JZ, Shen Y, Datta R, Abizaid A, Diano S, Horvath TL, Culler MD (2005) A novel growth hormone secretagogue-1a receptor antagonist that blocks ghrelin-induced growth hormone secretion but induces increased body weight gain. *Neuroendocrinology* 81:339-349.
- Hansson C, Haage D, Taube M, Egecioglu E, Salome N, Dickson SL (2011) Central administration of ghrelin alters emotional responses in rats: behavioural, electrophysiological and molecular evidence. *Neuroscience* 180:201-211.
- Harrold JA, Dovey T, Cai XJ, Halford JC, Pinkney J (2008) Autoradiographic analysis of ghrelin receptors in the rat hypothalamus. *Brain Res* 1196:59-64.
- Hashimoto H, Ueta Y (2011) Central effects of ghrelin, a unique peptide, on appetite and fluid/water drinking behavior. *Curr Protein Pept Sci* 12:280-287.
- Holst B, Cygankiewicz A, Jensen TH, Ankersen M, Schwartz TW (2003) High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. *Mol Endocrinol* 17:2201-2210.

- Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW (2004) Common structural basis for constitutive activity of the ghrelin receptor family. *J Biol Chem* 279:53806-53817.
- Hori Y, Kageyama H, Guan JL, Kohno D, Yada T, Takenoya F, Nonaka N, Kangawa K, Shioda S, Yoshida T (2008) Synaptic interaction between ghrelin- and ghrelin-containing neurons in the rat hypothalamus. *Regul Pept* 145:122-127.
- Hou Z, Miao Y, Gao L, Pan H, Zhu S (2006) Ghrelin-containing neuron in cerebral cortex and hypothalamus linked with the DVC of brainstem in rat. *Regul Pept* 134:126-131.
- Howard AD, Feighner SD, Cully DF, Arena JP, Liberators PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevich M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH (1996) A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273:974-977.
- Inhoff T, Monnikes H, Noetzel S, Stengel A, Goebel M, Dinh QT, Riedl A, Bannert N, Wissler AS, Wiedenmann B, Klapp BF, Tache Y, Kobelt P (2008) Desacyl ghrelin inhibits the orexigenic effect of peripherally injected ghrelin in rats. *Peptides* 29:2159-2168.
- Inhoff T, Wiedenmann B, Klapp BF, Monnikes H, Kobelt P (2009) Is desacyl ghrelin a modulator of food intake? *Peptides* 30:991-994.
- Ishida Y, Nakamura M, Ebihara K, Hoshino K, Hashiguchi H, Mitsuyama Y, Nishimori T, Nakahara D (2001) Immunohistochemical characterisation of Fos-positive cells in

- brainstem monoaminergic nuclei following intracranial self-stimulation of the medial forebrain bundle in the rat. *Eur J Neurosci* 13:1600-1608.
- Jaszberenyi M, Bujdoso E, Bagosi Z, Telegdy G (2006) Mediation of the behavioral, endocrine and thermoregulatory actions of ghrelin. *Horm Behav* 50:266-273.
- Jerlhag E (2008) Systemic administration of ghrelin induces conditioned place preference and stimulates accumbal dopamine. *Addict Biol* 13:358-363.
- Jerlhag E, Egecioglu E, Dickson SL, Andersson M, Svensson L, Engel JA (2006) Ghrelin stimulates locomotor activity and accumbal dopamine-overflow via central cholinergic systems in mice: implications for its involvement in brain reward. *Addict Biol* 11:45-54.
- Jerlhag E, Egecioglu E, Dickson SL, Douhan A, Svensson L, Engel JA (2007) Ghrelin administration into tegmental areas stimulates locomotor activity and increases extracellular concentration of dopamine in the nucleus accumbens. *Addict Biol* 12:6-16.
- Jerlhag E, Egecioglu E, Dickson SL, Engel JA (2010) Ghrelin receptor antagonism attenuates cocaine- and amphetamine-induced locomotor stimulation, accumbal dopamine release, and conditioned place preference. *Psychopharmacology (Berl)* 211:415-422.
- Jerlhag E, Egecioglu E, Landgren S, Salome N, Heilig M, Moechars D, Datta R, Perrissoud D, Dickson SL, Engel JA (2009) Requirement of central ghrelin signaling for alcohol reward. *Proc Natl Acad Sci U S A* 106:11318-11323.
- Jerlhag E, Engel JA (2011) Ghrelin receptor antagonism attenuates nicotine-induced locomotor stimulation, accumbal dopamine release and conditioned place preference in mice. *Drug Alcohol Depend* 117:126-131.

- Jerlhag E, Landgren S, Egecioglu E, Dickson SL, Engel JA (2011) The alcohol-induced locomotor stimulation and accumbal dopamine release is suppressed in ghrelin knockout mice. *Alcohol* 45:341-347.
- Jiang H, Betancourt L, Smith RG (2006) Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. *Mol Endocrinol* 20:1772-1785.
- Kaur S, Ryabinin AE (2010) Ghrelin receptor antagonism decreases alcohol consumption and activation of periculomotor urocortin-containing neurons. *Alcohol Clin Exp Res* 34:1525-1534.
- Kawahara Y, Kawahara H, Kaneko F, Yamada M, Nishi Y, Tanaka E, Nishi A (2009) Peripherally administered ghrelin induces bimodal effects on the mesolimbic dopamine system depending on food-consumptive states. *Neuroscience* 161:855-864.
- Kawakami A, Okada N, Rokkaku K, Honda K, Ishibashi S, Onaka T (2008) Leptin inhibits and ghrelin augments hypothalamic noradrenaline release after stress. *Stress* 11:363-369.
- Keen-Rhinehart E, Bartness TJ (2005) Peripheral ghrelin injections stimulate food intake, foraging, and food hoarding in Siberian hamsters. *Am J Physiol Regul Integr Comp Physiol* 288:R716-722.
- King SJ, Isaacs AM, O'Farrell E, Abizaid A (2011) Motivation to obtain preferred foods is enhanced by ghrelin in the ventral tegmental area. *Hormones and behavior* 60:572-580.
- Kinzig KP, Scott KA, Hyun J, Bi S, Moran TH (2006) Lateral ventricular ghrelin and fourth ventricular ghrelin induce similar increases in food intake and patterns of

- hypothalamic gene expression. *Am J Physiol Regul Integr Comp Physiol* 290:R1565-1569.
- Kirk RE (1982) *Experimental Design*. Belmont, CA: Wadsworth.
- Kobeissy FH, Jeung JA, Warren MW, Geier JE, Gold MS (2008) Changes in leptin, ghrelin, growth hormone and neuropeptide-Y after an acute model of MDMA and methamphetamine exposure in rats. *Addict Biol* 13:15-25.
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656-660.
- Kojima M, Kangawa K (2005) Ghrelin: structure and function. *Physiol Rev* 85:495-522.
- Koob G, Hicks MJ, Wee S, Rosenberg JB, De BP, Kaminsky SM, Moreno A, Janda KD, Crystal RG (2011) Anti-cocaine vaccine based on coupling a cocaine analog to a disrupted adenovirus. *CNS Neurol Disord Drug Targets* 10:899-904.
- Korotkova TM, Brown RE, Sergeeva OA, Ponomarenko AA, Haas HL (2006) Effects of arousal- and feeding-related neuropeptides on dopaminergic and GABAergic neurons in the ventral tegmental area of the rat. *Eur J Neurosci* 23:2677-2685.
- Lam DD, Garfield AS, Marston OJ, Shaw J, Heisler LK (2010) Brain serotonin system in the coordination of food intake and body weight. *Pharmacol Biochem Behav* 97:84-91.
- Landgren S, Engel JA, Andersson ME, Gonzalez-Quintela A, Campos J, Nilsson S, Zetterberg H, Blennow K, Jerlhag E (2009) Association of nAChR gene haplotypes with heavy alcohol use and body mass. *Brain Res* 1305 Suppl:S72-79.
- Landgren S, Jerlhag E, Hallman J, Orelund L, Lissner L, Strandhagen E, Thelle DS, Zetterberg H, Blennow K, Engel JA (2010) Genetic Variation of the Ghrelin

- Signaling System in Females With Severe Alcohol Dependence. *Alcohol Clin Exp Res* 34:1519-1525.
- Landgren S, Simms JA, Hyytia P, Engel JA, Bartlett SE, Jerlhag E (2012) Ghrelin receptor (GHS-R1A) antagonism suppresses both operant alcohol self-administration and high alcohol consumption in rats. *Addiction Biology* 17:86-94.
- Landgren S, Simms JA, Thelle DS, Strandhagen E, Bartlett SE, Engel JA, Jerlhag E (2011) The ghrelin signalling system is involved in the consumption of sweets. *PLoS One* 6:e18170.
- Leibowitz SF (1978) Paraventricular nucleus: a primary site mediating adrenergic stimulation of feeding and drinking. *Pharmacol Biochem Behav* 8:163-175.
- Leite-Moreira AF, Soares JB (2007) Physiological, pathological and potential therapeutic roles of ghrelin. *Drug Discov Today* 12:276-288.
- LeSauter J, Hoque N, Weintraub M, Pfaff DW, Silver R (2009) Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proc Natl Acad Sci U S A* 106:13582-13587.
- Lu SC, Xu J, Chinookoswong N, Liu S, Steavenson S, Gegg C, Brankow D, Lindberg R, Veniant M, Gu W (2009) An acyl-ghrelin-specific neutralizing antibody inhibits the acute ghrelin-mediated orexigenic effects in mice. *Mol Pharmacol* 75:901-907.
- Malik S, McGlone F, Bedrossian D, Dagher A (2008) Ghrelin modulates brain activity in areas that control appetitive behavior. *Cell Metab* 7:400-409.
- Mano-Otagiri A, Nemoto T, Sekino A, Yamauchi N, Shuto Y, Sugihara H, Oikawa S, Shibasaki T (2006) Growth hormone-releasing hormone (GHRH) neurons in the arcuate nucleus (Arc) of the hypothalamus are decreased in transgenic rats whose

- expression of ghrelin receptor is attenuated: Evidence that ghrelin receptor is involved in the up-regulation of GHRH expression in the arc. *Endocrinology* 147:4093-4103.
- Mietlicki EG, Daniels D (2011) Ghrelin reduces hypertonic saline intake in a variety of natriorexigenic conditions. *Exp Physiol* 96:1072-1083.
- Mietlicki EG, Nowak EL, Daniels D (2009) The effect of ghrelin on water intake during dipsogenic conditions. *Physiol Behav* 96:37-43.
- Miller DK, Nation JR, Wellman PJ (1999) Sensitization of anorexia and locomotion induced by chronic administration of ephedrine in rats. *Life sciences* 65:501-511.
- Morikawa H, Paladini CA (2011) Dynamic regulation of midbrain dopamine neuron activity: intrinsic, synaptic, and plasticity mechanisms. *Neuroscience* 198:95-111.
- Moulin A, Brunel L, Boeglin D, Demange L, Ryan J, M'Kadmi C, Denoyelle S, Martinez J, Fehrentz JA (2013) The 1,2,4-triazole as a scaffold for the design of ghrelin receptor ligands: development of JMV 2959, a potent antagonist. *Amino Acids* 44:301-314.
- Moulin A, Demange L, Berge G, Gagne D, Ryan J, Mousseaux D, Heitz A, Perrissoud D, Locatelli V, Torsello A, Galleyrand JC, Fehrentz JA, Martinez J (2007a) Toward potent ghrelin receptor ligands based on trisubstituted 1,2,4-triazole structure. 2. Synthesis and pharmacological in vitro and in vivo evaluations. *J Med Chem* 50:5790-5806.
- Moulin A, Ryan J, Martinez J, Fehrentz JA (2007b) Recent developments in ghrelin receptor ligands. *ChemMedChem* 2:1242-1259.

- Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M, Kangawa K (2002) Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. *J Endocrinol* 174:283-288.
- Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S (2001) A role for ghrelin in the central regulation of feeding. *Nature* 409:194-198.
- Naleid AM, Grace MK, Cummings DE, Levine AS (2005) Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. *Peptides* 26:2274-2279.
- Ogiso K, Asakawa A, Amitani H, Inui A (2011) Ghrelin: a gut hormonal basis of motility regulation and functional dyspepsia. *J Gastroenterol Hepatol* 26 Suppl 3:67-72.
- Olds J, Milner P (1954) Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *J Comp Physiol Psychol* 47:419-427.
- Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, Hayflick L, Butler RN, Allison DB, Ludwig DS (2005) A potential decline in life expectancy in the United States in the 21st century. *N Engl J Med* 352:1138-1145.
- Palmiter RD (2007) Is dopamine a physiologically relevant mediator of feeding behavior? *Trends Neurosci* 30:375-381.
- Paxinos G, Watson C (2004) *The Rat Brain in Stereotaxic Coordinates*. Amsterdam: Elsevier Academic Press.
- Perello M, Sakata I, Birnbaum S, Chuang JC, Osborne-Lawrence S, Rovinsky SA, Woloszyn J, Yanagisawa M, Lutter M, Zigman JM (2010) Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. *Biol Psychiatry* 67:880-886.

- Petersen PS, Woldbye DP, Madsen AN, Egerod KL, Jin C, Lang M, Rasmussen M, Beck-Sickinger AG, Holst B (2009) In vivo characterization of high Basal signaling from the ghrelin receptor. *Endocrinology* 150:4920-4930.
- Pomerleau CS, Saules K (2007) Body image, body satisfaction, and eating patterns in normal-weight and overweight/obese women current smokers and never-smokers. *Addict Behav* 32:2329-2334.
- Pomerleau OF (1986) Nicotine as a psychoactive drug: anxiety and pain reduction. *Psychopharmacol Bull* 22:865-869.
- Quarta D, Di Francesco C, Melotto S, Mangiarini L, Heidbreder C, Hedou G (2009) Systemic administration of ghrelin increases extracellular dopamine in the shell but not the core subdivision of the nucleus accumbens. *Neurochem Int* 54:89-94.
- Repaci A, Gambineri A, Pagotto U, Pasquali R (2011) Ghrelin and reproductive disorders. *Mol Cell Endocrinol* 340:70-79.
- Richardson KA, Aston-Jones G (2012) Lateral hypothalamic orexin/hypocretin neurons that project to ventral tegmental area are differentially activated with morphine preference. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32:3809-3817.
- Ruter J, Kobelt P, Tebbe JJ, Avsar Y, Veh R, Wang L, Klapp BF, Wiedenmann B, Tache Y, Monnikes H (2003) Intraperitoneal injection of ghrelin induces Fos expression in the paraventricular nucleus of the hypothalamus in rats. *Brain Res* 991:26-33.
- Salome N, Haage D, Perrissoud D, Moulin A, Demange L, Egecioglu E, Fehrentz JA, Martinez J, Dickson SL (2009a) Anorexigenic and electrophysiological actions of novel ghrelin receptor (GHS-R1A) antagonists in rats. *Eur J Pharmacol* 612:167-173.

- Salome N, Hansson C, Taube M, Gustafsson-Ericson L, Egecioglu E, Karlsson-Lindahl L, Fehrentz JA, Martinez J, Perrissoud D, Dickson SL (2009b) On the central mechanism underlying ghrelin's chronic pro-obesity effects in rats: new insights from studies exploiting a potent ghrelin receptor antagonist. *J Neuroendocrinol* 21:777-785.
- Santos GC, Marin MT, Cruz FC, Delucia R, Planeta CS (2009) Amphetamine- and nicotine-induced cross-sensitization in adolescent rats persists until adulthood. *Addiction Biology* 14:270-275.
- Scott V, McDade DM, Luckman SM (2007) Rapid changes in the sensitivity of arcuate nucleus neurons to central ghrelin in relation to feeding status. *Physiol Behav* 90:180-185.
- Sellings LH, McQuade LE, Clarke PB (2006) Evidence for multiple sites within rat ventral striatum mediating cocaine-conditioned place preference and locomotor activation. *The Journal of pharmacology and experimental therapeutics* 317:1178-1187.
- Shimbara T, Mondal MS, Kawagoe T, Toshinai K, Koda S, Yamaguchi H, Date Y, Nakazato M (2004) Central administration of ghrelin preferentially enhances fat ingestion. *Neurosci Lett* 369:75-79.
- Shrestha YB, Wickwire K, Giraud S (2009) Effect of reducing hypothalamic ghrelin receptor gene expression on energy balance. *Peptides* 30:1336-1341.
- Skibicka KP, Dickson SL (2011) Ghrelin and food reward: the story of potential underlying substrates. *Peptides* 32:2265-2273.

- Skibicka KP, Hansson C, Alvarez-Crespo M, Friberg PA, Dickson SL (2011a) Ghrelin directly targets the ventral tegmental area to increase food motivation. *Neuroscience* 180:129-137.
- Skibicka KP, Hansson C, Egecioglu E, Dickson SL (2011b) Role of ghrelin in food reward: impact of ghrelin on sucrose self-administration and mesolimbic dopamine and acetylcholine receptor gene expression. *Addict Biol.*
- Smith AM, Pivavarchyk M, Wooters TE, Zhang Z, Zheng G, McIntosh JM, Crooks PA, Bardo MT, Dwoskin LP (2010) Repeated nicotine administration robustly increases bPiDDB inhibitory potency at alpha6beta2-containing nicotinic receptors mediating nicotine-evoked dopamine release. *Biochem Pharmacol* 80:402-409.
- Spinedi E, Voirol MJ, Verdumo C, Giacominni M, Pralong F, Gaillard RC (2006) Hypothalamic ghrelin treatment modulates NPY-but not CRH-ergic activity in adrenalectomized rats subjected to food restriction: Evidence of a novel hypothalamic ghrelin effect. *Endocrine* 29:477-484.
- Sun Y, Ahmed S, Smith RG (2003) Deletion of ghrelin impairs neither growth nor appetite. *Mol Cell Biol* 23:7973-7981.
- Sun Y, Butte NF, Garcia JM, Smith RG (2008) Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. *Endocrinology* 149:843-850.
- Szentirmai E, Kapas L, Krueger JM (2007) Ghrelin microinjection into forebrain sites induces wakefulness and feeding in rats. *Am J Physiol Regul Integr Comp Physiol* 292:R575-585.

- Szentirmai E, Kapas L, Sun Y, Smith RG, Krueger JM (2009) Restricted feeding-induced sleep, activity, and body temperature changes in normal and preproghrelin-deficient mice. *Am J Physiol Regul Integr Comp Physiol* 298:R467-477.
- Takahashi T, Ida T, Sato T, Nakashima Y, Nakamura Y, Tsuji A, Kojima M (2009) Production of n-octanoyl-modified ghrelin in cultured cells requires prohormone processing protease and ghrelin O-acyltransferase, as well as n-octanoic acid. *J Biochem* 146:675-682.
- Takano S, Kim J, Ikari Y, Ogaya M, Nakajima K, Oomura Y, Wayner MJ, Sasaki K (2009) Electrophysiological effects of ghrelin on laterodorsal tegmental neurons in rats: an in vitro study. *Peptides* 30:1901-1908.
- Tessari M, Catalano A, Pellitteri M, Di Francesco C, Marini F, Gerrard PA, Heidbreder CA, Melotto S (2007) Correlation between serum ghrelin levels and cocaine-seeking behaviour triggered by cocaine-associated conditioned stimuli in rats. *Addict Biol* 12:22-29.
- Tschop M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. *Nature* 407:908-913.
- Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, Folwaczny C (2001) Post-prandial decrease of circulating human ghrelin levels. *J Endocrinol Invest* 24:RC19-21.
- van Furth WR, van Ree JM (1996) Sexual motivation: involvement of endogenous opioids in the ventral tegmental area. *Brain Res* 729:20-28.
- van Zessen R, van der Plasse G, Adan RA (2012) Contribution of the mesolimbic dopamine system in mediating the effects of leptin and ghrelin on feeding. *Proc Nutr Soc* 1-11.

- Wellman PJ, Clifford PS, Rodriguez JA, Hughes S, Di Francesco C, Melotto S, Tessari M, Corsi M, Bifone A, Gozzi A (2012) Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fMRI and intracranial self-stimulation. *Addiction Biology* 17:908-919.
- Wellman PJ, Davis KW, Nation JR (2005) Augmentation of cocaine hyperactivity in rats by systemic ghrelin. *Regul Pept* 125:151-154.
- Wellman PJ, Elliott AE, Barbee S, Hollas CN, Clifford PS, Nation JR (2008a) Lobeline attenuates progressive ratio breakpoint scores for intracranial self-stimulation in rats. *Physiology & behavior* 93:952-957.
- Wellman PJ, Hollas CN, Elliott AE (2008b) Systemic ghrelin sensitizes cocaine-induced hyperlocomotion in rats. *Regul Pept* 146:33-37.
- Williams DL, Grill HJ, Cummings DE, Kaplan JM (2003) Vagotomy dissociates short- and long-term controls of circulating ghrelin. *Endocrinology* 144:5184-5187.
- Wise RA (1996) Addictive drugs and brain stimulation reward. *Annual review of neuroscience* 19:319-340.
- Wise RA (2002) Brain reward circuitry: insights from unsensed incentives. *Neuron* 36:229-240.
- Wise RA (2004) Dopamine, learning and motivation. *Nat Rev Neurosci* 5:483-494.
- Wise RA, Leeb K (1993) Psychomotor-stimulant sensitization: a unitary phenomenon? *Behav Pharmacol* 4:339-349.
- Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR (2001a) Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 86:5992.

- Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR (2001b) Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50:2540-2547.
- Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR (2000) The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 141:4325-4328.
- Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL (2008) Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 132:387-396.
- Yannielli PC, Molyneux PC, Harrington ME, Golombek DA (2007) Ghrelin effects on the circadian system of mice. *J Neurosci* 27:2890-2895.
- Yeomans JS (1989) Two substrates for medial forebrain bundle self-stimulation: myelinated axons and dopamine axons. *Neurosci Biobehav Rev* 13:91-98.
- Zago A, Leao RM, Carneiro-de-Oliveira PE, Marin MT, Cruz FC, Planeta CS (2012) Effects of simultaneous exposure to stress and nicotine on nicotine-induced locomotor activation in adolescent and adult rats. *Braz J Med Biol Res* 45:33-37.
- Zan Y, Haag JD, Chen KS, Shepel LA, Wigington D, Wang YR, Hu R, Lopez-Guajardo CC, Brose HL, Porter KI, Leonard RA, Hitt AA, Schommer SL, Elegbede AF, Gould MN (2003) Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. *Nat Biotechnol* 21:645-651.
- Zigman JM, Elmquist JK (2006) In search of an effective obesity treatment: a shot in the dark or a shot in the arm? *Proc Natl Acad Sci U S A* 103:12961-12962.

Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK (2006) Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol* 494:528-548.

Zimmermann US, Buchmann A, Steffin B, Dieterle C, Uhr M (2007) Alcohol administration acutely inhibits ghrelin secretion in an experiment involving psychosocial stress. *Addict Biol* 12:17-21.

Zorrilla EP, Iwasaki S, Moss JA, Chang J, Otsuji J, Inoue K, Meijler MM, Janda KD (2006) Vaccination against weight gain. *Proc Natl Acad Sci U S A* 103:13226-13231.