Orexigenic peptides and drug-related behavior: A role for neuropeptide Y and ghrelin

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ABSTRACT

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It has been often noted that natural and drug rewards show similar behavioral characteristics, and share some of the underlying neural pathways. The orexigenic peptides, neuropeptide-Y (NPY) and ghrelin, traditionally involved in homeostatic regulation of feeding, have been shown to have a significant modulatory impact on drug-related behaviors. In the experiments described in the present thesis, we first used an animal model of relapse to drug abuse, the reinstatement model, to assess the role of NPY Y1 and Y5-receptor-mediated transmission in food deprivation (FD)-induced reinstatement of heroin seeking. Results demonstrated that injections of a novel NPY Y5-receptor antagonist, Lu AA33810 (0.0, or 30.0/kg/IP), resulted in a significant attenuation of FD-induced reinstatement of extinguished heroin seeking. However no effects were found for the Y1-receptor antagonist, BIBO-3304 (0.0, 5.0, or 10.0 nmol/ICV) or the Y5-receptor antagonist, L-152-804 (0.0, or 20.0 μg/ICV). We then studied the role of ghrelin in on-going heroin self-administration and FD-induced reinstatement of extinguished heroin seeking. Surprisingly, although infusions of ghrelin (0.0, 1.5, and 3.0 μg/rat, i.c.v.) produced increases in breakpoints on a progressive ratio schedule of reinforcement, antagonism of ghrelin receptors had no effect on on-going heroin self-administration, or on FD-induced reinstatement of heroin seeking. These results suggest that while signals mediated through NPY Y1 and ghrelin receptors play a modest role in drug reinforcement and reinstatement, activation of Y5-receptors has a critical function in FD-induced reinstatement of heroin seeking behavior.

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TABLE OF CONTENTS

OREXIGENC PEPTIDES AND DRUG-RELATED BEHAVIOR: A ROLE FOR NEUROPEPTIDE-Y AND GHRELIN

Severe perturbations of mechanisms that mediate the rewarding effects of stimuli such as food or drugs of abuse could ultimately lead to maladaptive patterns of eating seen in patients that suffer from obesity and anorexia or bulimia nervosa, and compulsive drug taking and seeking observed in drug addicts. It has been often noted that natural and drug rewards show similar behavioral characteristics, and share some of the underlying neural pathways. Moreover, it has been shown that these different types of rewarding stimuli have modulatory effects on one another, and thus it is proposed that they are part of a cooperative "reward" system. Evidence for this potentially shared system comes from studies demonstrating that drugs of abuse alter the choice and amount of food consumed in humans and animals (Heffner, Zigmound & Stricker, 1977), and can activate and modify neuronal circuitry that mediates food reward or feeding-related processes (Carroll 1985; Carr, 1996; Volkow & Wise, 2005).

Given the evidence that drugs of abuse can alter mechanisms that control food intake, it has been postulated that the inverse relationship also exists: that a shift in homeostatic mechanisms controlling natural reward processes like feeding, can also alter mechanisms that mediate the effects of drugs of abuse (Carr, 2007). For example, consumption of highly palatable foods, periods of food scarcity and metabolic manipulations have been shown to alter drug related behavior (Liu & Grigson, 2005; Franklin, Schiele, Brozek, & Keys, 1948; Shalev, Highfield, Yap, & Shaham, 2000). Accordingly, numerous studies (e.g., Harris, Wimmer, & Aston-

Jones, 2005; Wellman, Davis, & Nation, 2005; Shalev, Yap, & Shaham, 2001) have explored this relationship by investigating how manipulations in hormonal or peptidergic systems known to be involved in the regulation of food intake, can modulate drug-taking and drug-seeking behavior. The goal of the present thesis was to extend findings from previous studies showing that peptides traditionally known for their functions in energy homeostasis are also associated with drug-related behavior. Specifically, the role of ghrelin and neuropeptide-Y was assessed during drug-self-administration and reinstatement of drug-seeking behavior, animal models of drug-taking behavior and relapse, respectively.

Animal models of drug-taking behavior and relapse

Intravenous drug self-administration

Drug addiction is a chronic condition characterized by compulsive drug taking with periods of both abstinence and relapse (O'Brien & McLellan, 1996; Koob & Le Moal, 2008). It is estimated that approximately 0.8% of the population in Canada (Statistics Canada, 2002) and 1.56% of Americans (National Survey on Drug use and Health, 2008) are dependent upon illicit drugs. Unfortunately, treatment remains unsuccessful mostly due to high incidences of relapse to drug seeking, and eventual drug taking behavior following prolonged periods of abstinence. In fact, it is estimated that 40-60% of recovering addicts relapse (McLellan, Lewis, O'Brien & Kleber, 2000); therefore drug addiction is a phenomenon that merits serious attention.

Drug dependence is a multi-faceted and complex disease and thus requires a multidimensional approach. Both human and animal research is necessary in examining drug addiction and relapse, as they provide different levels of investigation

into the disease. The utility of animal models has been demonstrated by important findings uncovering both behavioral mechanisms and neurobiological substrates of addiction.

Animal models of reward seeking per se are extensive, well validated and robust. Although many models have proved to be useful, i.e. intracranial self-stimulation (ICSS), and conditioned place preference (CPP), the most common model in studies of the reinforcing effects of drugs of abuse is the self-administration model. The self-administration model requires an animal to perform an operant behavior such as a lever press in order to obtain rewarding stimuli (food, sucrose pellets, or drugs of abuse) either intravenously or orally. This model has face, predictive and construct validity displayed by 1) drug-taking itself, 2) the capacity of animals to self-administer the same drugs of abuse as humans and 3) the similarities of neurobiological substrates found in the laboratory and in natural settings (Panlilio & Goldberg, 2007). Thus, the self-administration model is predictive of addictive potential. Moreover, self-administration can include valuable procedures such as adjustments in rate of responding following changes in demand, drug doses and second-order conditioning, which allows for an assessment of the reward value of drugs of abuse. One procedure of particular importance is the progressive ratio (PR) schedule of reinforcement. This schedule is considered as a better assessment of the animals' motivation to obtain the drug reward than a fixed ratio schedule of reinforcement. Unlike the fixed ratio schedule of reinforcement where responses are reinforced after an unchanging number of emitted responses, in the progressive ratio schedule, response requirements increase exponentially with each consecutive drug

infusion. A critical feature of the PR is its breakpoint, the point at which the animal is no longer willing to perform the target behavior to obtain the reward (Roberts & Bennett, 1993). This point provides an index of motivational and reinforcing effects of drugs of abuse and natural rewards (Stafford, LeSage, & Glowa, 1998). The utility of the PR schedule of reinforcement in assessing motivation can be observed in its ability to assess reward strength or reinforcer efficacy, demonstrated by increased breakpoint found when higher doses of rewarding stimuli such as sucrose, are introduced (Roberts & Bennett, 1993; Baron, Mikorski, & Schlund, 1992; Stafford & Branch, 1998). Thus, it is apparent that the self-administration model via different experimental procedures provides a good model to demonstrate the rewarding, reinforcing and motivational effects of drugs of abuse.

The reinstatement model: Drug-seeking behavior following periods of extinction

In the United States, there are 2.4 million persons presently undergoing treatment in rehabilitation facilities (National Survey on Drug Use and Health, 2009), however, unfortunately, relapse still occurs in 40-60% of abstinent addicts (McLellan et al., 2000). Therefore, the factors that underlie human relapse warrant critical investigation in order to reduce the potential for future lapses and to provide appropriate relapse interventions. Three factors are known to provoke relapse in humans: re-exposure to drugs of abuse, drug-associated cues, and stressful events (Epstein, Preston, Stewart & Shaham, 2006). However, due to ethical and practical issues associated with studying relapse in humans, an animal model, which investigates similar relapsing factors, has been developed and termed "the

reinstatement procedure". The model involves a period of drug self-administration training, followed by a period of drug-seeking-behavior extinction training. Subsequently, while in a drug-free state, animals are re-exposed to the drug (de Wit & Stewart, 1981; 1983), drug-associated cues (de Wit & Stewart, 1981), or acute stressors (Shaham & Stewart, 1995), and their restored drug-seeking responding is evaluated. This model has been used to investigate both the environmental conditions and the underlying neuronal processes associated with relapse and has been found to be valid and reliable across species of animals such as rodents and non-human primates (Epstein et al., 2006).

Of the triggers known to elicit relapse, one of particular importance is stress, as human drug abusers have reported stress or stressful events as the basis for the initiation, maintenance and relapse to drug use (Brewer, Catalano, Haggerty, Gainey & Fleming, 1998; Matheney & Weatherman, 1998; Sinha, 2001) and thus much effort has been made to uncover the effects of stress on drug taking and drug seeking behavior in animal models. Traditionally, animal models of stress-induced relapse have implemented extreme environment stressors (e.g. electrical foot-shock), which have little ecological validity. In contrast, a period of acute food deprivation is one stressor thought to be the most ecologically relevant to environmental conditions found to elicit relapse in humans (Lu, Shepard, Hall & Shaham, 2003).

Food deprivation and reward-related behavior

Early observations have shown that dysregulation in metabolic processing such as a state of negative energy balance can impact reward-related behavior. The most rudimentary disruption of energy homeostasis could be found during periods of

war, where food deficit is most prominent. For example, anecdotal evidence has shown that when World War II conscientious objectors were exposed to experimental semi-starvation, their nicotine and caffeine (Franklin et al., 1948) increased. Moreover, a sample of Peruvian Indians displayed increased cocoa leaf chewing when they were malnourished, whereas when they were provided with well-balanced meals, their leaf chewing decreased (Hanna & Hornick, 1977).

In laboratory animals, it is known that dietary manipulations, such as food deprivation and food restriction, can modulate neurobiological reward systems, and therefore alter the rewarding, reinforcing, conditioned, and locomotor-activating properties of food as well as of addictive drugs. Particularly, it is well established that both food restriction (FR) and food deprivation (FD) can facilitate drug-related behaviors across drugs of abuse such as amphetamine, cocaine and morphine (Bell, Stewart, Thompson & Meisch, 1997; Deroche, Piazza, Casolini, Le Moal & Simon, 1993), routes of administration (i.e. oral, intravenously, systemically or intracranially; Carroll, France & Meisch, 1979; Cabeza de Vaca & Carr, 1998), and species such as rodents and primates (Comer, Lac, Wyvell, Curtis & Carroll, 1995; Rodefer & Carroll 1996; Comer, Turner & Carroll, 1995). However it is important to note that these metabolic manipulations have distinct properties. Typically, chronic food restriction refers to limiting access to food over a period of several days or weeks or until an animal reaches a target body weight ranging between 75-95% of free-feeding body weight or by providing a limited access to a food quota (5-8 g). In contrast, the food deprivation procedure involves the complete removal of access to food for 21-23 hours (Lu et al., 2003). Initial studies showed that animals that were exposed to food

restriction regimes resulting in a reduction to 80% of free-freeing body weight demonstrated a robust increase in cocaine (Carroll, 1985; Papasava, Oei & Singer, 1981), amphetamine (Takahashi, Singer, & Oei, 1978), heroin and methadone (Oei, Singer & Jeffreys, 1980) self-administration, and expressed reinstatement of extinguished cocaine-seeking (Carroll, 1985) and augmented drug-priming-induced reinstatement of extinguished responding for cocaine (Comer, et al., 1995). Similarly, food deprivation (23-24 hours) has been shown to potentiate the initiation of intravenous cocaine self-administration (Oei, 1983) and induce reinstatement of heroin and cocaine seeking (Shalev et al., 2000; Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003). Moreover, prolonged periods of food deprivation (up to 4 days) have been shown to augment the locomotor-activating effects of amphetamine (Campbell & Fibiger, 1971). Together, these findings suggest that food restriction and acute food deprivation can augment the rewarding, conditioned, and locomotor activating effects of psychoactive drugs.

Modulation of reward by peripheral and central signals associated with the control of feeding

Peripheral feeding-regulating signals such as leptin, an anorexigenic signal released by adipose tissue that is involved in long-term energy balance (Friedman & Halaas, 1998), and ghrelin, an orexigenic peptide that is produced by the glands of the stomach during fasting states (Kojima et al., 1999), modulate central peptide transmission in the arcuate nucleus of the hypothalamus (ARC). Leptin increases the activity of anorexigenic cells in the brain and the release of peptides like cocaine-and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) which

result in the inhibition of feeding, while inhibiting cells that release orexigenic peptides like agouti-related protein (AgRP) and neuropeptide Y (NPY), which initiate feeding. Ghrelin, on the other hand, has the opposite action and inhibits anorexigenic neurons (i.e. CART and POMC) and activates orexigenic neurons (i.e. AgRP and NPY; Abizaid, Gao, & Horvath, 2006a).

These peripheral and central signals involved in energy balance have been demonstrated to also be involved in the modulation of reward-related processes. For example, the anorexigenic peptide, leptin, is thought to be involved in processing the rewarding properties of food, as demonstrated by an attenuation of sucrose self-administration (Figlewicz, Bennett, Naleid, Davis, & Grimm, 2006) and conditioned place preference for high-fat diets (Figlewicz et al., 2004) in leptin-treated rats. This notion has been supported by a study showing that ICSS into particular sites in the lateral hypothalamus that are sensitive to food-restriction can be attenuated by intracerebroventricular (ICV) infusions of leptin (Fulton, Woodside & Shizgal, 2000). Central infusions of leptin can also alter the conditioned properties of drugs-associated cues, as demonstrated by the attenuation of food-deprivation induced reinstatement of heroin seeking (Shalev et al., 2001). This suggests that adiposity-associated anorexigenic signaling is an important component in the processing of reward related stimuli. Therefore, it is proposed that other peripheral signals (i.e. ghrelin) and hypothalamic hormones (i.e. NPY) which function as orexigenic mechanisms, might also be involved in the rewarding properties of food and drugs of abuse.

Neuropeptide Y (NPY)

It has been well-established that NPY can powerfully induce feeding (Levine, Jewett, Cleary, Kotz & Billington, 2004). NPY is a 36 amino-acid member of the pancreatic polypeptide family, and is the most abundant peptide in the mammalian central nervous system, with the highest concentration found in the hypothalamus and brainstem (Allen et al., 1983). NPY neurons are found in the ARC, which is also innervated by NPY neurons arising from the brainstem (Sahu, Kalra, Crowley & Kalra, 1988). Importantly, NPY neurons project to the paraventricular nucleus of the hypothalamus (PVN), ventromedial hypothalamus (VMH), and the dorsomedial nucleus of the hypothalamus (DMH), all areas that regulate ingestive behavior. It has been demonstrated that an increase in ARC NPY gene expression and increased NPY release in the PVN occurs following food deprivation (Bi, Robinson & Moran, 2003). Therefore, it was suggested that NPY provides a central signal for the initiation of food intake and reduced energy expenditure (Sahu, Kalra & Kalra, 1988). This is supported by findings that show that exogenous NPY robustly increases food intake and body weight in rats when administered ICV (Clark, Kalra, Crowley & Kalra, 1984) and intra-PVN (Stanley & Leibowitz, 1984), as well as fat accumulation, demonstrated by white fat lipoprotein lipase gene expression in food-restricted rats (Billington, Briggs, Grace & Levine, 1991), indicating a regulatory role of NPY to compensate for metabolic challenges. Interestingly, NPY (ICV) has also been found to increase feeding during the light period of the light/dark cycle, when rats usually do not eat (Levine & Morley, 1984), and in sated rats (Stanley & Lebowitz, 1985). Moreover, the effect of NPY on food intake has been found to be more potent than food deprivation itself (Jewett, Cleary, Levine, Schaal, & Thompson, 1995). For

example, NPY has been found to be more potent in increasing breakpoints on a PR schedule of food reinforcement than insulin and acute food deprivation (Jewett et al., 1995), indicating a strong effect on the increase in the motivation to obtain food. This suggests that the orexigenic effects of NPY could occur beyond a homeostatic need, and could reflect an augmentation of the rewarding value of food.

In addition to its involvement in food reward and appetitive reinforcement, NPY has been hypothesized to be involved in mediating the rewarding or reinforcing potential of addictive drugs. In support of this notion, we have recently demonstrated that ICV NPY administration enhanced cocaine-induced hyperactivity and moderately increased cocaine self-administration (Maric, Cantor, Cuccioletta, Tobin, & Shalev, 2009). Furthermore, heroin self-administration and reinstatement of extinguished drug seeking in heroin-trained animals were enhanced following ICV NPY treatment (Maric, Tobin, Quinn, & Shalev, 2008).

While the effects of NPY on drug reward have been extensively discussed (Maric et al., 2008; 2009), the involvement of specific NPY receptors in the drug-self administration and reinstatement paradigm has yet to be investigated. Six NPY receptor subtypes (Y1-Y6) have been identified. Of these, the Y1 and Y5 receptor subtypes have been most frequently associated with ingestive behavior in mammals (Eva, Serra, Mele, Panzica & Oberto, 2006). Intracerebroventricular administration of NPY Y1-receptor antagonists decreases food deprivation induced-feeding (Kask, Rajo, & Harro, 1998a) and intra-PVN injection inhibits NPY induced feeding and hyperphagia induced by 24 hour fast in rats (Wieland, Engel, Eberlein, Rudolf & Doods, 1998). Moreover, injections of NPY Y5-receptor agonist ICV have been

shown to produce increases in food intake (McCrea et al., 2000) and chronic administration resulted in obesity-like body weights (Mashiko et al., 2003). In contrast, food intake and body weight is reduced by blockade of NPY Y5-receptor in diet-induced obese mice (Ishihara et al., 2005). Interestingly, these receptors have been implicated in mediating the psychoactive effects of drug. For example, NPY Y1-receptor antagonists inhibit the locomotor enhancing effects of amphetamine (Kask & Harro, 2000), and activation of NPY Y5-receptors has been shown to reduce morphine withdrawal precipitated by the opioid antagonist, naloxone (Woldbye, Kelmp & Madsen, 1998).

Ghrelin

Ghrelin is a recently discovered 28 amino acid peptide that is an endogenous agonist to the growth hormone secretagogue receptor (GHS-R). Ghrelin is mostly synthesized in the stomach, and is then released into the blood stream (Kojima et al., 1999), and subsequently crosses the blood-brain area to target relevant brain sites to elicit orexigenic actions. Plasma ghrelin levels elevate during periods of fasting and quickly drop once feeding occurs (Sanchez, Oliver, Pico & Palou, 2004). Likewise, exogenous ghrelin administration both peripherally and centrally produces hyperphagia in food-deprived rats (Nakazato et al., 2001) and acts as a "hunger signal", presumably mimicking interoceptive cues produced by periods of food deprivation (Davidson et al., 2005). Ghrelin is also synthesized in hypothalamic nuclei such as the DMH, VMH, and PVN (Cowley et al., 2003). The central activation of hypothalamic ghrelin receptors via circulating ghrelin due to emptying of the gut and from intra-hypothalamic ghrelin release, is the mechanism that

stimulates the experience of pre-prandial hunger and consequently leads to the initiation of feeding. Specifically, these orexigenic effects are mediated through the activation of ghrelin receptors that are co-localized with NPY and AgRP neurons in the arcuate nucleus of the hypothalamus (Horvath, Diano, Sotonyi, Heiman & Tschop, 2001). Furthermore, GHS-R antagonists have been found to inhibit feeding in lean, diet-induced obese, and ob/ob obese mice, animals that are genetically mutated to lack the ability to produce leptin and thus have uncontrolled mechanisms of feeding (Asakawa et al., 2003).

Although ghrelin's involvement in the regulation of energy balance is evident, ghrelin may also increase the rewarding and reinforcing value of food, which would motivate an organism to approach rewarding stimuli. For example, exogenous administration of ghrelin either systemically or centrally, increases food-intake in sated rats (Wren et al., 2000; Hashimoto et al., 2007), induces feeding during periods where minimal amount of feeding occurs (Sanchez et al., 2004) and induces hyperphagia in lean animals (Beck, Richy & Stricker-Krongrad, 2004). These findings suggest that the function of ghrelin extends beyond energy homeostasis possibly by affecting hedonic and/or motivational aspects of feeding, and potentially influencing other rewarding stimuli. Evidence for a hedonic role for ghrelin comes from studies demonstrating that acute injections of ghrelin modulate acquisition of conditioned place preference (CPP) for high-fat diets in mice (Perello et al., 2009), augment behavioral effects of cocaine, such as hyperactivity (Wellman et al., 2005), and also enhance rewarding properties of cocaine as measured by conditioned place preference in rats (Davis, Wellman & Clifford, 2007).

Together, these findings indicate that orexigenic signals involved in energy balance (i.e. ghrelin and NPY signaling) might intercede the rewarding effects of food, and importantly, also of drugs of abuse.

Rationale for current experiments

Food deprivation modulates NPY transmission in the hypothalamus via peripheral anorexigenic or orexigenic signaling, namely, leptin and ghrelin. Thus, their effects on drug-related behavior, such as the attenuation of food deprivation-induced reinstatement (by leptin; Shalev et al., 2001), and augmentation of cocaine-induced CPP and hyperactivity (by ghrelin; Davis et al., 2007), might be mediated by NPY transmission. Support for this view is found in our previous finding, where NPY administration resulted in increased heroin self-administration and reinstatement of heroin-seeking (Maric et al., 2008). The current study was designed in order to reveal whether orexigenic hormones are *necessary* for the expression of drug-related behavior. First, we hypothesized that if food deprivation-induced increases in NPY transmission is critically involved in food-deprivation-induced reinstatement of drug seeking, treatments with specific NPY Y1 and Y5 receptors antagonists would attenuate this effect. Second, it was hypothesized that like NPY, ICV injections of ghrelin could mimic the effects of food deprivation/restriction on the motivational properties of heroin, as demonstrated by changes in on-going self-administration on a PR schedule of reinforcement. Finally, it was expected that ghrelin antagonism would

modulate the rewarding properties of heroin as demonstrated by changes in on-going heroin self-administration, and would attenuate food-deprivation-induced reinstatement of extinguished heroin seeking.

EXPERIMENT 1. Effects of NPY Y1 receptor antagonism on food-deprivation induced reinstatement of heroin seeking

Experiment 1A. The effects of the NPY Y1-receptor antagonist, BIBO 3304, on food-deprivation induced reinstatement of heroin seeking

Method

Subjects

All rats were treated in accordance with the Canadian Council on Animal Care and approval was granted by the Concordia University Animal Care Committee. Thirty Long-Evans male rats (Charles River, Raleigh, NC, USA; 300-350g) were used. Prior to surgery, they were housed in pairs in the animal care facility during which time they were handled daily. Following recovery from intravenous (IV) and intracerebroventricular guide cannulation (ICV) surgery, animals were housed in operant chambers under a reversed light/dark cycle (Lights off at 9:30 a.m, on at 9:30 p.m.), throughout the experiment. *Ad libitum* access to water and food was available, except for the 21 hr food deprivation condition. Body weights were measured daily.

Surgery

To allow for drug self-administration animals were implanted with intravenous Silastic catheters (Dow Corning, Midland, MI, USA). Prior to surgery each animal was anesthetized with a mixture of xylazine and ketamine (10 + 100 mg/kg, intra peritoneal (IP)) and given penicillin (450,000 IU/rat) to prevent infection. Once the rat was fully anesthetized, as confirmed by the absence of a withdrawal reflex to a paw-pinch, two incisions were made: one on the skull and one on the neck (approximately 1 cm above the ventral side of the shoulder blade). Tweezers were inserted through the neck incision and the jugular vein was isolated. A small incision was made on the jugular vein's surface allowing for the insertion of a 3 cm catheter. The catheter was then secured to the vein with silk sutures and the remaining portion of the catheter was directed subcutaneously to the top of the skull where it was attached to a modified 22-gauge cannula (Plastics One Industries, Roanoke, VA). Immediately following catheterization, a 22-gauge cannula aimed 2 mm above the right lateral ventricle (AP - 0.5, ML +1.4, DV -3.0, relative to bregma) was implanted to allow for the ICV injections of NPY Y1-receptor antagonist. Both cannulae were subsequently anchored to the skull with dental cement and jeweler screws. Post surgery, animals were given the analgesic buprenorphine (0.01 mg/kg/rat; Schering-Plough Ltd., Welwyn Garden City Hertfordshire, UK), to prevent pain. Following recovery from surgery, guide cannula placement was verified by demonstrating a short latency (<60s) drinking response to angiotensin II (100 nmol, ICV). Throughout the experiment, the catheters were flushed daily with heparin and the antibiotic drug gentamicin in saline solution (7.5 IU + 12.0 mg/rat) to prevent

blockage and infection of the catheters. Animals that did not flush post-surgery were excluded from the experiment.

Apparatus

Throughout the experiment, the animals were individually housed in two types of operant self-administration chambers (Med Associates, St. Albans, VT, and Coulbourn Instruments, Allentown, PA, USA; 32.0 cm x 24.0 cm x 25.0 cm; 29.0 cm x 24.0 cm x 25.5 cm, respectively), which had identical designs. Both chambers were enclosed in sound attenuating wooden compartments furnished with fans for ventilation and diminishment of noise. Operant chambers comprised of stainless steel grid floor, two front and back transparent Plexiglas walls, and two side metal panel walls. Each chamber had 'active' and 'inactive' levers, which were placed approximately 5 cm from the floor. The 'inactive' lever remained extended thoughout the experiment, and presses did not result in any programmable consequences and were intended to assess nonspecific activity and response generalization. The 'active' lever extended at the beginning of each session and responses on that lever activated the infusion pump, resulting in drug infusions. Drug infusions were paired with illumination of a cue light above the active lever for 20s (Coulbourn Instruments) and activation of a tone module (Sonalert, 2.9 KHz, Coulbourn Instruments). Rats were attached to the infusion pump via a liquid swivel (Instech, Boulder, CO, USA) and a polyethylene-50 tubing shielded with a metal spring.

Infusions, active and inactive lever presses were recorded daily with Graphic State Software (Coulbourn Instruments) on a computer connected to the operant chambers via Link boxes (Coulbourn Instruments). ICV injections of NPY Y1-

receptor antagonist were performed using a microinfusion pump (Harvard Apparatus *11 plus*, Holliston, MA) fitted with a 10 µl Hamilton syringe (Hamilton Company, Reno, NV). The injector (28-gauge) extended 2 mm below the implanted guide cannula and was kept in place for another 60 s after the injection to allow for complete diffusion of the NPY Y1- receptor antagonist.

Drugs

Heroin HCL (National Institute for Drug Abuse, Baltimore, MD) was dissolved in sterile saline (5 mg/ml), which was then further diluted with 0.9% saline solution according to body weight for a dose of 0.1mg/kg/infusion. NPY Y1-receptor antagonist BIBO 3304 ((R)-N-{[4-aminocarbonylaminomethyl)-phenyl]methyl}-N2-(diphenylacetyl)-argininamide trifluoroacetate; TOCRIS, MO) was dissolved in dimethyl sulfoxide (DMSO) and distilled water to a concentration of either 2.5 nmol/ul or 5.0 nmol/ul. The antagonist was delivered in a volume of 2 µl to reach final doses of 5.0 nmol or 10.0 nmol/rat/ICV.

Procedure

Self-administration training. Following a 24 hr habituation period in the operant chamber, the rats were trained to self-administer heroin with three 3 hr self-administration sessions per day under a fixed ratio 1 (FR-1) schedule of reinforcement for a total of 10-12 days. During this period, one press on the active lever resulted in one infusion. Each day, the session began approximately 1 hr after the onset of the dark phase of the light/dark cycle (approximately 10:00 a.m.). At the beginning of the session the inactive and active levers extended into the chambers. Simultaneously, the cue-light above the active lever was activated and the tone

sounded for 30 s. Subsequently, each response on the active lever resulted in the delivery of 0.1 mg/kg of heroin over 5 s, and the initiation of a 20 s timeout period. During the timeout period, lever presses were not reinforced, the houselight was turned off, and the cue-light/tone complex was turned on. At the end of each 3 hr session the active lever was retracted but the inactive lever remained in the chamber, this allowed animals to further discriminate between the lever associated with the drug reward and the one with no reinforcing value.

Extinction. Following 10-12 days of training, animals were submitted to extinction training that followed the same procedure as the training days, but consisted of only one 3 hr session/day, with the exception of the first day of extinction, which consisted of three 3 hr sessions/day. During extinction, heroin syringes were removed and thus presses on the 'active lever' previously associated with the drug no longer resulted in drug delivery. Extinction training continued for a minimum of 4 days and until animals reached an extinction criterion of 15 or less active lever presses per 3 hr session before conducting reinstatement tests.

Reinstatement Test. Once animals met extinction criterion, they were exposed to two 3 hr reinstatement test sessions, which were preceded by either 21 hr of food-deprivation (food hoppers removed) or 21 hr of unlimited access to food, in a counterbalanced order. Rats were injected with the NPY Y1-receptor antagonist BIBO 3304 (0.0, or 5.0 or 10.0 nmol/ rat, ICV) 15 min before each test. BIBO 3304 was injected ICV over a period of 4 min using a 28-gauge injector that extends 3 mm below the tip of the guide cannula. The injector was retained in place for an additional 60 s after the injection to ensure the dispersion of the drug. Approximately 30 min

following food-deprivation tests, food hoppers were returned. Each test session was followed by and preceded by a minimum of two baseline days.

Statistical Analyses

Reinstatement data were analyzed with separate mixed design ANOVAs, which were conducted for each dependent variable (active and inactive responses) with a within-subjects factor of *condition* (baseline, 21 hr food deprivation (FD) and *ad libitum (*AL)), and a between-subjects factor of BIBO 3304 *dose* (0.0, 5.0, or 10.0 nmol/rat/ICV). Baseline condition was calculated by averaging the last of day of extinction before the reinstatement tests. Simple effects were followed up with Fisher's LSD post-hoc comparisons. The critical cut-off point for significant results was set at $p \leq 0.05$.

Results

Food intake Test. A separate set of animals (n=10) were habituated to powdered food in the animal facility to evaluate the efficacy of the of NPY Y1-receptor antagonist at 1 hr, 3 hr and 24 hr post-injection. Treatment with a high dose (10.0 nmol/rat/ICV) of NPY Y1-receptor antagonist (M=3.15, SE=0.58) reduced amount of food intake compared to baseline *(*M=4.33, SE=0.42) and vehicle (M=4.25, SE=0.42) conditions at 1 hr post-injection (See APPENDIX A, Table 1., for 3 hr and 24 hr results).

Self-administration Training. Animals demonstrated greater number of presses on the 'active' drug-paired lever relative to the 'inactive lever' non-drug paired lever throughout self-administration training, indicating a learned association between the active lever and drug reinforcement (Figure 1).

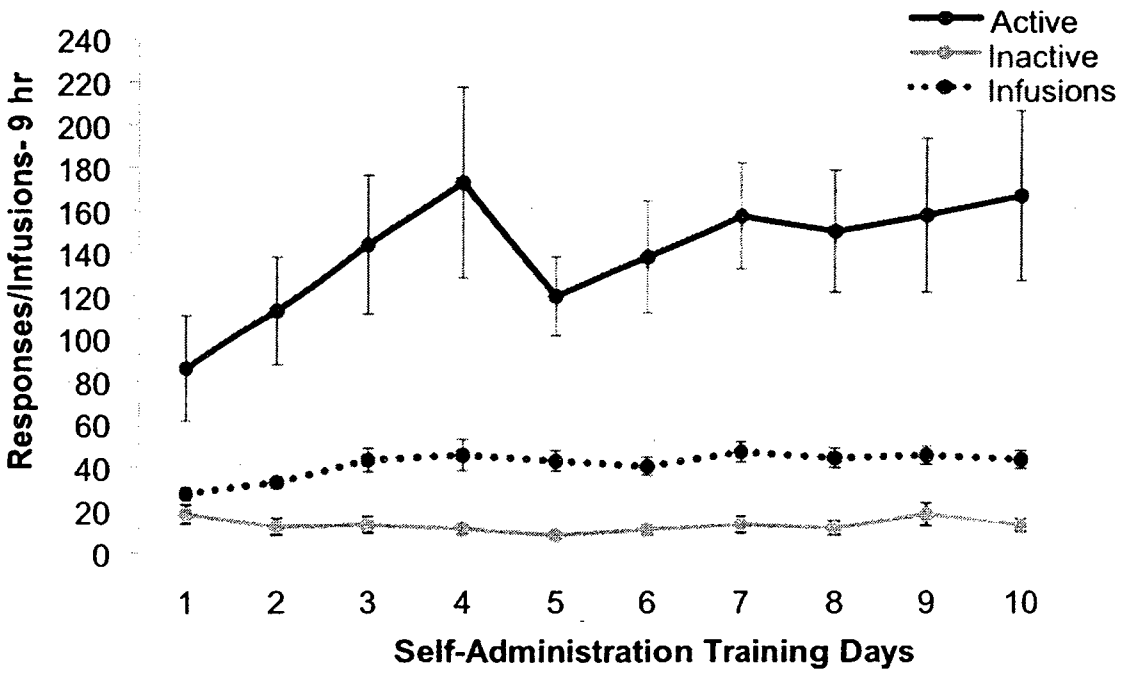

Figure 1. Heroin self-administration training. Mean (+/-*SE*) number of active lever presses, inactive lever presses and heroin infusions (0.10 mg/kg/infusion) over a 10-day training period (three 3 hr sessions, $n = 23$).

Reinstatement Test. Following training and extinction, animals were subjected to one of three BIBO 3304 doses (0.0, 5.0, 10.0 nmol/rat/ICV). Seven animals were removed due to catheter blockage (n=5), illness (n=1) or poor self-administration training (n=1). Final analyses included data from 23 rats in three *BIBO 3304 dose* groups, 0.0 nmol (n=10), 5.0 nmol (n=4) and 10.0 nmol (n=9). No significant interaction between *condition* and *BIBO 3304 dose* was found [$F(4, 40)=1.37$, $p=.26$]. Active presses in the food deprivation condition (M=51.00, SE=8.79) were higher compared to baseline (M=10.44, SE=0.72) and *ad libitum* (M=17.31, SE=2.19) conditions, resulting in a significant main effect for *condition* [$F(2, 20)=16.32$, $p<.001$]. No significant main effect for active presses was found between BIBO 3304 doses [$F(2,20)=2.37$, $p=.12$]. Due to a significant difference found for the *condition* factor, a one-way ANOVA on data from the FD condition was conducted. Results revealed a statistical trend ($p= .084$), where the 5.0 nmol BIBO 3304 dose resulted in an increase in active presses, as compared to vehicle (0.0 nmol) treated animals within the food-deprivation condition. No significant effects for inactive lever presses were found, all p's > .05 (Figure 2).

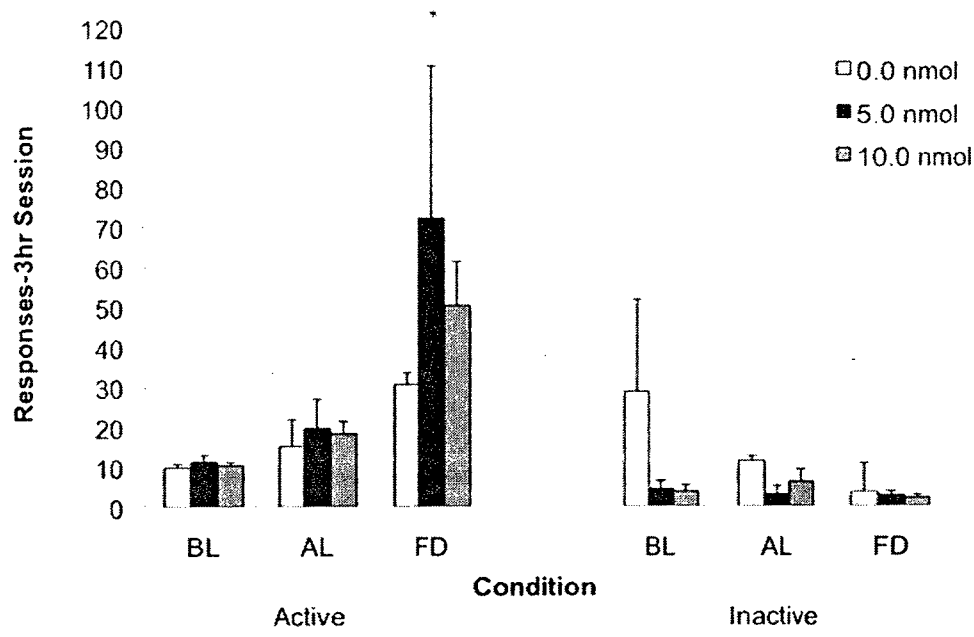

Figure 2. The effect of NPY Y1-receptor antagonist BIBO 3304, on FD-induced reinstatement of heroin seeking. Data presented are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), and following NPY Y1-receptor antagonist injection (0.0, 5.0 or 10.0 nmol/rat/ICV) 15 min before the sessions, on the 21 hr FD, or *ad libitum* (AL) conditions. Baseline condition (BL) represents mean responding on the last day of extinction prior to each test session. * Significant difference ($p < 0.05$) between the FD condition and the baseline and AL conditions.

Summary

Results from experiment 1A demonstrated that NPY Y1-receptors are not critically involved in FD-induced reinstatement of heroin-seeking under extinction conditions. It is suggested that although NPY Y1-receptors modulate food and drug reward it appears that they play a minimal role in mediating the conditioned properties of drug reward-associated cues. Moreover, the findings presented here suggest that Y1 receptor antagonism might result in augmentation of FD-induced reinstatement of heroin seeking. Since NPY, mainly through the Y1 receptors, has anxiolytic properties, and Y1-receptor antagonists have been shown to be anxiogenic (Heilig, 2004), the effect of BIBO 3304 on anxiety levels in food-deprived rats was further assessed.

Experiment 1B. The effects of Y1 receptor antagonism on performance in the elevated plus maze in food deprived rats.

Method

Subjects

Following experiment 1A, animals (n= 10) were housed in the animal facility on the same reversed light/dark cycle (Lights off at 9:30 a.m., on at 9:30 p.m.) for a period of 2 weeks for drug washout and habituation. *Ad libitum* access to water and food was available, except for the 21 hr food deprivation condition. Body weights were measured daily.

Surgery

As described in experiment 1A, animals were previously implanted with a 23-gauge guide cannula aimed 2 mm above the right lateral ventricle (AP - 0.5, ML +1.4, DV -3.0, relative to bregma) to allow for the ICV injections of the NPY Y1-receptor antagonist.

Elevated Plus Maze Apparatus

Located in a dimly lit room, the maze was made out of wood and consisted of four arms (11.5 x 55.0 cm) positioned at right angles 50 cm above the floor. The maze comprised of two arms considered as "Closed" arms, which had 40 cm high walls and two "Open" arms, which had 1.0 cm high ledges.

Drugs

BIBO 3304 (10.0 nmol/rat/ICV) was injected ICV at a volume of 2 µl over a period of 4 min using a 28-gauge injector that extends 3 mm below the tip of the guide cannula.

Procedure

Habituation. Animals were handled daily for 1 week prior to the elevated plus maze experiment. Two consecutive habituation days were conducted for animals to become accustomed to ICV injection procedure, animal transportation, environmental conditions in the testing room and the elevated plus maze apparatus. On each habituation day animals received sham ICV injections in the animal facility (at 9:30

a.m.) where they were attached to microinfusion pump, the pump was turned on for 4 minutes, however no infusions were given. They were then brought into the testing room from the animal facility and were placed on the elevated plus maze for 10 minutes of exploration. Animals were placed on the center platform with their head facing a closed arm. Once the habituation period was complete, fecal boli were collected and counted, and the maze was wiped down with 70% ethanol. Animals were then transported back to the animal facility.

Elevated Plus Maze Test. Immediately following the last habituation day, all animals were food-deprived for 21 hr, by removing all food from the cages. Water was available for this period *ad libitum.* Rats were assigned to either the BIBO 3304 (10.0 nmol/rat/ICV, n=5) or vehicle (0.0 nmol/rat/ICV, n=5). Ten minutes following the injection animals were brought from the animal facility to the testing room and were placed on the maze for 10 min. Following the maze test fecal boli was collected and counted, and the maze was wiped down with 70% ethanol, to ensure environmental neutrality.

Plus Maze Scoring. Habituation and test days were video-recorded and then scored by a blind observer who was unaware of treatment assignment. Four variables were observed and scored as the following: Time spent on closed arms (TC): when all paws are in the closed arm, two hind paws are in closed but two front paws are in middle or on open arm; Time spent on open arms (TO): when all paws are on the open arm, when two paws and more than half the body are stretched onto open arm; Number of open arm approaches (OA): open arm approaches from center form, peeking from the closed arm with body stretching into the open arm, full entry into

the open arm; Number of closed arm entries (CE): number of closed arm entries from open arm and crossings into each of closed arms from other closed arm.

Statistical Analyses

Elevated plus maze data were analyzed using separate One-Way ANOVAs with the between-subjects factor *of BIBO 3304 dose* (0.0 or 10.0 nmol/rat/ICV) for the four dependent variables described above (TC, TO, OA, CE). The critical cut-off point for significant results was set at $p \leq 0.05$. Although number of fecal boli was recorded, no statistical analysis was conducted, as most rats did not display any. However, it is interesting to note that animals that did display excretion (n=2) were animals treated with BIBO 3304 (10.0 nmol/rat/ICV).

Results

Results demonstrated a statistically significant effect for BIBO 3304 treatment on TC [$F(1,8)=5.37$, $p=.041$] with BIBO 3304 treated rats spending more time in the closed arms (M=81.40, SE=4.40) compared to vehicle treated animals (M=55.60, SE=9.64; Figure 3). No significant differences were found for number of closed entries (CE) [$F(1,9)=3.70$, $p=.090$] between BIBO 3304 (M=9.2, SE=0.97) and vehicle treated animals (M=7.2, SE=0.37; See APPENDIX B for open arm data).

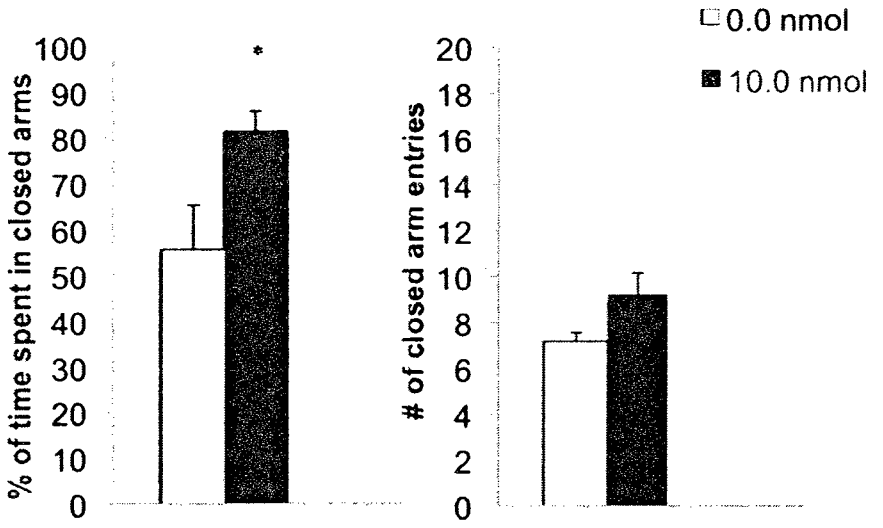

Figure 3. The effect of the NPY Y1-receptor antagonist BIBO 3304, on anxiety as measured on the elevated plus maze in food-deprived (21 hr) rats. Data presented are mean (+*SE*) for percentage of time spent in the closed arms (left) and number of closed arm entries (left) following NPY Y1-receptor antagonist injection (0.0, or 10.0 nmol/rat/ICV; $n = 5$ per group) * $p < 0.05$ compared to vehicle.

Summary

The present findings demonstrated that Y1 receptor antagonism using BIBO-3304, at doses known to inhibit feeding, augments anxiety-induced behavior as demonstrated on the elevated plus maze. These findings, together with findings from experiment 1A, suggest that although Y1 receptors are not critical for FD-induced reinstatement of drug-seeking, they perhaps play a role in opposing the stressful effects of FD, as NPY-Y1 activation has been shown to have anxiolytic properties (Heilig, 2004). Considering that hypothalamic NPY mRNA is elevated during periods of food restriction and deprivation (Bi et al., 2003), and that NPY Y1-receptors do not appear to be important for the expression of FD-induced reinstatement of drug seeking, it is possible that the effects of NPY on drug-related behaviors is mediated via other NPY receptor subtypes.

EXPERIMENT 2: Effects of NPY Y5 receptor antagonism on food-deprivation induced reinstatement of heroin seeking

Method

Subjects

Fifty Long-Evans male rats (Charles River, Raleigh, NC, USA; 300-350g) were used. Prior to surgery, they were housed in pairs in the animal care facility during which time they were handled daily. Following recovery from surgery, animals were housed in operant chambers under a reversed light/dark cycle (Lights off at 9:30 a.m., on at 9:30 p.m.), where they resided for the entirety of the experiment. During experiment 2A, animals had unlimited access to water, and food

was made available *ad libitum* at the end of the first three hours of self-administration, except for the 48 hr food deprivation condition, to minimize associations made between food availability and drug. Food hoppers were returned approximately 30 minutes after the end of the first 3-hr session during self-administration training, extinction and reinstatement baseline days. During experiment 2B, *ad libitum* access to water and food was available, except for the 21 hr food deprivation condition. Body weights were measured daily.

Surgery

Surgery procedures were identical to those previously described in experiment 1A. Rats were implanted with intravenous silastic catheters into the right jugular vein to allow for drug self-administration. The catheter was secured to the vein with a silk suture and passed subcutaneously to the top of the skull where it was attached to a modified 22-gauge cannula. For experiment 2A, a unilateral 22-gauge cannula was also implanted during the intravenous surgery aimed 2 mm above the right lateral ventricle (AP - 0.5, ML +1.4, DV -3.0, relative to bregma) to allow for ICV injections of the Y5-Antagonist L-152-804.

Apparatus

Experiments were conducted in two types of operant self-administration chambers (Med Associates and Coulbourn Instruments) as described in experiment 1A.

Drugs.

Heroin HCL (National Institute for Drug Abuse, Baltimore, MD) was dissolved in sterile saline (5 mg/ml), which was then further diluted with 0.9% saline according to body weight for a dose of 0.1mg/kg/infusion. NPY Y5-receptor antagonist L-152-804 (TOCRIS Bioscience, MO; Experiment 2A) was dissolved in 10% dimethyl sulfoxide (DMSO) and 45% cyclodextrin in sterile saline to a concentration of 5 μg/ul and delivered at a volume of 4 μl/min to reach a dose of 0.0 μg/rat/ICV or 20.0 μg/rat/ICV. NPY Y5-antagonist Lu AA33810 (Lundbeck Research, NJ) was dissolved in 5% DMSO, 1% methylcellulose and sterile saline and administered IP at a dose of 0.0 mg/kg or 30 mg/kg.

General Procedure.

Self-Administration Training. Following one day of habituation and auto shaping, self-administration training was conducted over 10-12 days, with three 3 hr sessions per day. All other aspects of self-administration training were identical as those described in experiment 2.

Extinction. Following 10-12 days of training, animals were submitted to extinction training, during which time the drug was removed, yet animals continued to be exposed to all contextual and response-contingent cues for a minimum of 4 days and until they reached the extinction criterion of 15 active lever presses or less per session. Beginning on the second day of extinction training, animals received sham injections during which time they were connected to an activated microinfusion pump but were not injected (experiment 2A), or received injections of saline (0.5 ml, IP;

experiment 2B). This established a baseline condition to control for behavioral differences resulting from injection procedures.

Reinstatement Test. Once animals met the extinction criterion, they were exposed to two 3 hr reinstatement test sessions.

Experiment 2A. Reinstatement tests were preceded by either 48 hr FD (food hoppers removed) or 48 hr of unlimited access to food, in a counterbalanced order. During FD or AL conditions, rats were given an "off" period where no extinction sessions were run. Rats remained in the self-administration chambers but levers were not extended, cue lights were inactivated and the houselight remained off for the 48 h prior to reinstatement testing. The following day, 15 minutes before each test, rats were injected with the NPY Y5-antagonist L-152- 804 (0.0μg or 20.0μg/rat ICV) and the injector was retained in place for an additional 60 s after the injection to ensure the dispersion of the drug.

Experiment 2B. Reinstatement tests were preceded by either 21 hr FD (food hoppers removed) or 21 hr of unlimited access to food, in a counterbalanced order. Thirty minutes before each test, rats were injected with NPY Y5-antagonist Lu AA33810 (0.0 mg/kg or 30mg/kg/rat IP).

Each test session was followed by and preceded by a minimum of two baseline days.

Statistical Analyses.

Reinstatement data were analyzed by using a mixed design ANOVA for each experiment (A & B) with a within-subjects factor of *condition* (baseline, food deprivation and *ad libitum*), and a between subject factor of *NPY-Y5 antagonist dose*

(152-804; 0.0 µg or 20.0 µg/rat ICV, experiment 2A; Lu AA33810; 0.0 mg/kg or 30.0 mg/kg IP, experiment 2B). Baseline conditions were calculated by averaging the last of day of extinction before the reinstatement tests. Simple effects were followed up with Fisher's LSD post-hoc comparisons. The critical cut-off point for significant results was set at $p \leq .05$.

Results

Food intake Test. Prior to the experiment, separate sets of animals were habituated to powdered food in the animal facility to evaluate the efficacy of the antagonists at 1 hr, 3 hr and 24 hr of allowed feeding time.

NPY-Y5 antagonist L-152- 804 (n=3). Treatment with *NPY Y5-receptor antagonist* (20.0 µg/rat/ICV; *M*=2.17, *SE*=0.93) resulted in a slight reduction in the amount of food intake compared to baseline *(M*=3.08, *SE*=0.98) and vehicle (0.0 µg/rat/ICV; *M*=3.17, *SE*=0.83) conditions at 1 hr post-injection (See APPENDIX A, Table 2., for 3 hr and 24 hr results).

NPY-Y5 antagonist Lu AA33810. Animals were pre-treated with one of two antagonist doses Lu AA33810 (0.0 mg/kg or 30.0 mg/kg IP), which was followed, 15 minutes later, by Y5-agonist [cPP$^{1-7}$, NPY $^{19-23}$, Ala ^{31}, Aib ^{32}, Gin^{34}]-hPancreatic Polypeptide (0.6nmol/rat/ICV; SIGMA-ALDRICH, MO) for all animals. Inhibition of Y5-agonist-induced feeding via Y5-antagonism was investigated. NPY Y5-receptor antagonist reduced cPP-induced food intake (*M*=5.75, *SE*=1.03; *n*=4) compared to vehicle (*M*=10.67, *SE*=2.33; *n*=3) treated animals at 1 hr feeding time (See APPENDIX A, Table 3., for 3 hr and 24 hr results).

Experiment 2A. Effects of NPY Y5-receptor antagonist, L-152-804, on food-deprivation induced reinstatement of heroin-seeking

Self-administration Training. Animals demonstrated greater number of presses on the 'active' drug-paired lever relative to the 'inactive lever' non-drug paired lever throughout self-administration training, indicating a learned association between the active lever and drug reinforcement (Figure 4).

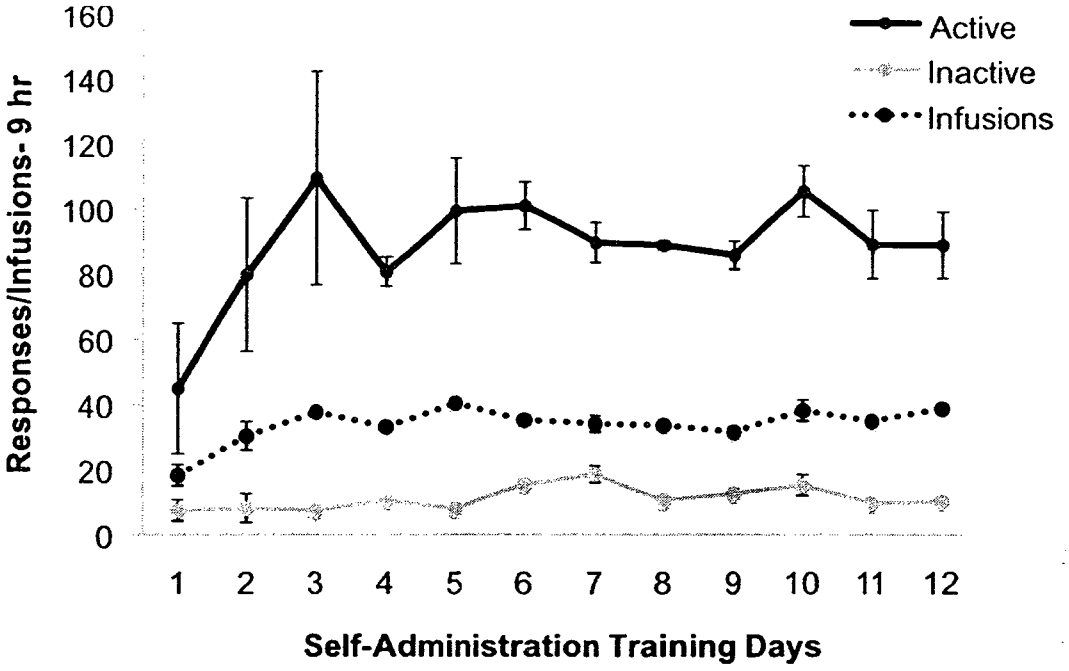

Figure 4. Heroin self-administration training. Mean (+/-*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.10 mg/kg/infusion) over the 12-day training period (three 3 hr sessions/day, $n = 14$).

Reinstatement Test. Six animals were removed due to catheter leak or blockage (n=3), illness (n=1) or poor self-administration training (n=1). One rat was removed from the analysis because of an extreme response during the *ad libitum* condition with the Y5 antagonist dose of 20.0 µg/rat/ICV (71 active lever responses compared to a mean of 11.57). Final analyses included data from 14 rats in two treatment groups of *L-152-804 dose*, 0.0 µg/rat/ICV (n=7), or 20.0 µg/rat/ICV (n=7). Results revealed no significant interaction between *condition* and *L-152-804 dose* for active presses, [$F(2, 24)=0.82, p=.922$]. A significant main effect for *condition* on active lever presses, [$F(1, 12)=8.37, p=.002$] was found, where active presses in the food deprivation condition (M=36.07, SE=8.26) were higher compared to baseline (M=10.24, SE=1.49) and *ad libitum* (M=9.42, SE=1.70) conditions regardless of treatment. No significant main effect between *L-152-804 doses* 0.0µg/rat (M=18.548, SE=3.78) and 20.0 µg/rat (M=19.63, SE=3.708), was found for active lever presses [$F(1,12)=0.042, p=.841$]. No significant effects for inactive lever presses were found, all p's > .05 (Figure 5).

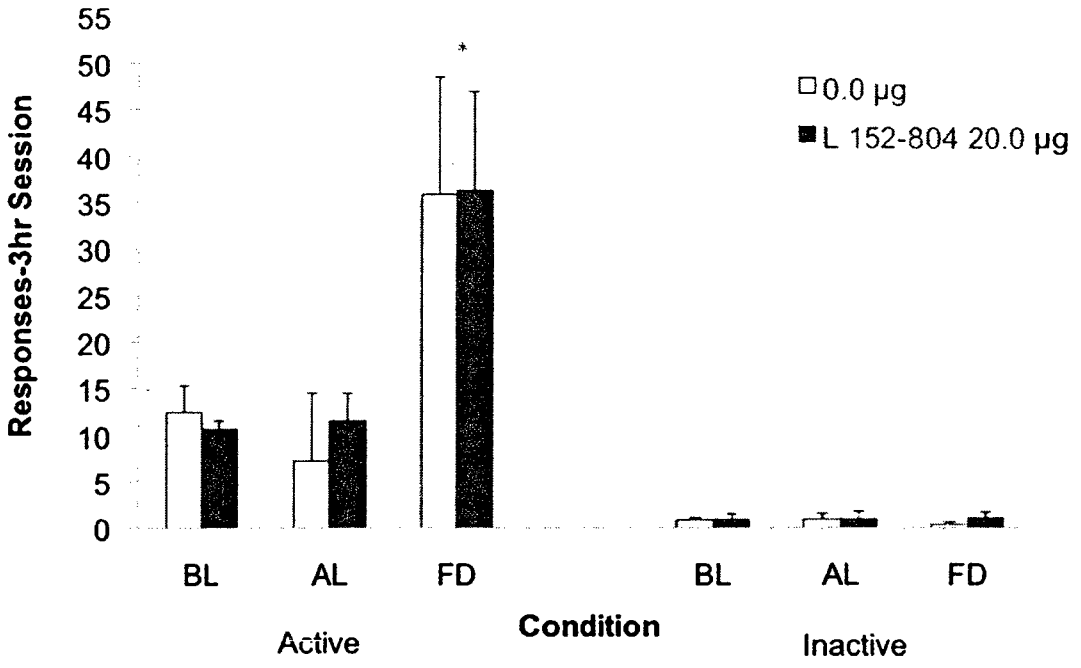

Figure 5. The effect of NPY Y5-receptor antagonist L-152-804, on 48 hr FD-induced reinstatement of heroin seeking. Data presented are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), and following L-152-804 injection (0.0 or 20.0 µg/rat/ICV) 15 min before the session, on the 48 hr FD, or *ad libitum* (AL) test days. Baseline condition (BL) represents mean responding on the last day of extinction prior to each test session. * Significant difference ($p < .05$) between the FD condition and the baseline and AL conditions.

Experiment 2B. Effects of the NPY Y5-receptor antagonist, Lu AA33810, on food-deprivation induced reinstatement of heroin-seeking

Self-administration Training. Animals demonstrated greater number of presses on the 'active' drug-paired lever relative to the 'inactive lever' non-drug paired lever throughout self-administration training, indicating a learned association between the active lever and drug reinforcement (Figure 6).

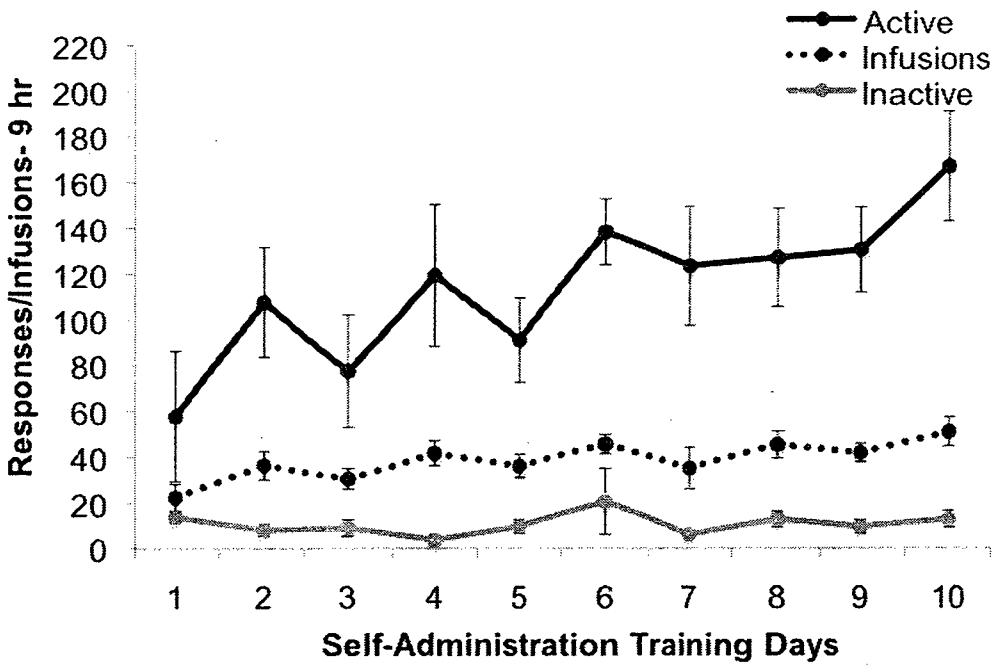

Figure 6. Heroin self-administration training. Mean (+/-*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.10 mg/kg/infusion) over the 10-day training period (three 3h sessions/day, $n = 18$).

Reinstatement Test. Two animals were removed due to illness. Final analyses included data from 18 rats in two treatment groups of *Lu AA33810 dose*, 0.0 mg/kg/rat/IP (n=8), and 30.0 mg/kg/rat/IP (n=10). A statistically significant interaction between *condition* and *Lu AA33810 dose* for active presses, [F(2, 32)=5.04, p=.012] was found, reflecting an attenuation of FD-induced reinstatement by Lu AA33810 (M= 18.10, SE= 10.63), as compared to vehicle treated animals (M=54.375, SE=11.89). A significant main effect for *condition* on active lever presses, [F(1, 16)=10.20, p=.002] was found, where active presses in the food deprivation condition (M=36.24, SE=7.98) were higher compared to baseline (M=9.70, SE=0.693) and *ad libitum* (M=15.40, SE=4.69) conditions regardless of treatment. A main effect approaching statistical significance [F(1, 16)=3.29, p=.09] for *Lu AA33810 dose,* reflecting higher number of active lever presses in the 0.0 mg/kg/rat (M=27.70, SE=5.96) compared to the 30.0 mg/kg/rat dose (M=13.18, SE=5.33), was found. No significant effects for inactive lever presses were found, all p's > .05 (Figure 7).

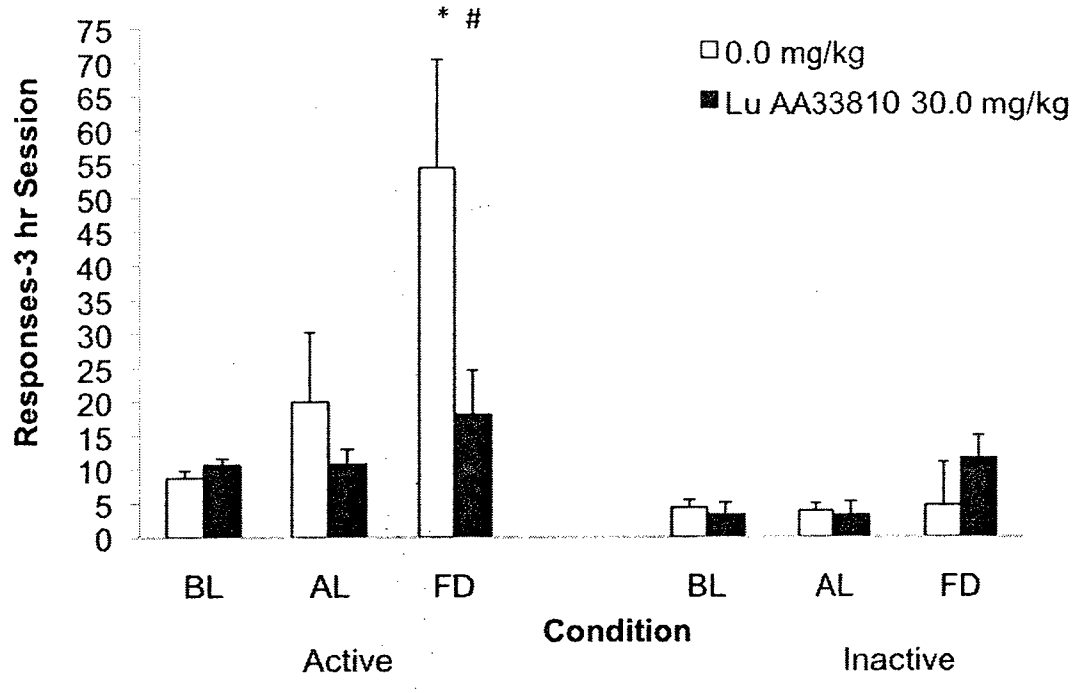

Figure 7. The effect of NPY Y5-receptor antagonist Lu AA33810, on 21 hr FD-induced reinstatement of heroin seeking. Data presented are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), and following Lu AA3810 injection (0.0 or 30.0 mg/kg/rat/IP) 30 min before the session on the 21 hr FD, or *ad libitum* (AL) test days. Baseline condition (BL) represents mean responding on the last day of extinction prior to each test session. * $p < 0.05$ vehicle-FD compared to BL and AL conditions # $p<0.05$ vehicle-FD compared to Lu AA33810-FD.

Summary

Results from experiment 2A demonstrated that the Y5-receptor antagonist L-152-804, although previously found to have high affinity to the Y5 receptor (Kanatani et al., 2000), did not modulate FD-induced reinstatement of drug seeking. In contrast, in experiment 2B, Lu AA33810, a novel, more potent antagonist with high affinity to Y5 receptors (Walker et al., 2009), demonstrated a significant attenuation of FD-induced reinstatement. Thus, it appears that Y5 receptors are imperative for the expression of FD-induced reinstatement of previously extinguished drug-seeking behavior. Since NPY transmission, through the Y5-receptor as suggested here, modulates drug-related behavior, we hypothesized that activation of ghrelin receptors which stimulates NPY release (Cowley et al., 2003), would also be involved in drug-related behavior. In order to investigate this hypothesis, we first examined whether ghrelin can augment the reinforcing and conditioned effects of drugs of abuse, as was previously shown with NPY (Maric et al., 2008).

EXPERIMENT 3: Effects of ghrelin infusions on breakpoints on a progressive ratio schedule of heroin reinforcement

Method

Subjects

Subjects were 20 rats (Charles River, St. Constant, QC) that weighed 275-300 g at the beginning of the experiment. Prior to surgery, the rats were housed in pairs for 1 week, in the animal facility at Concordia University, under a normal light dark cycle (Lights on at 9:00AM, off at 9:00PM). Once the animals reached a bodyweight

of approximately 350 g, IV catheterization and ICV cannulation surgery was performed. Following surgery, the animals were singly housed in plastic shoebox cages for another 48 hr after which they were transferred to the self-administration operant chambers. They remained in the operant chambers until the end of the experiment. With the exception of food removal during the drug self-administration sessions, animals were given *ad libitum* access to food and water.

Surgery

Surgical procedures were identical to those described in experiment 1, where animals were implanted with intravenous silastic catheters into the right jugular vein to allow for drug-self-administration. A unilateral 22-gauge guide cannula aimed 2mm above the right lateral ventricle (AP - 0.5, ML +1.4, DV -2.0, relative to bregma) was implanted to allow for the ICV injections of ghrelin.

Apparatus

Throughout the experiment, the animals were individually housed in one of 10 identical operant chambers (29.0 cm x 29.0 cm x 25.5 cm; Coulbourn Instruments, Allentown, PA, USA). All other aspects of the apparatus were identical to experiments previously described.

Drugs

Heroin (National Institute for Drug Abuse, Baltimore, MD) was dissolved in sterile distilled water to produce a stock solution of 5 mg/ml, which was then further diluted with 0.9% saline solution (BACHEM, CA) according to body weight to a dose of 0.1 mg/kg/infusion. Ghrelin was dissolved with physiological saline to concentrations of .75 μg/μl and 1.5 μg/μl delivered at a volume of 2 μl. The final dose

was 1.5 μg or 3.0 μg/rat/ICV. The doses selected have been shown to reliably induce feeding behavior (Wellman, Hollas & Elliot, 2008).

Procedure

Following one day of habituation where the animals were brought to the self-administration chambers and allowed to freely explore the chamber for 24 hr, self-administration training was conducted with daily single 5-hr session for 5-10 days under FR-1 schedule of reinforcement, and under a progressive ratio (PR) for another 12 days.

FR-1 self-administration training. During this training period, one press on the active lever resulted in one infusion. Each day, the session began approximately 1 hr after the onset of the light phase (approximately 10:00 a.m.). Food hoppers were removed during the self-administration session and returned approximately 1 hr after the session was completed in order to avoid any association between the return of the food and the end of the self-administration session. At the beginning of the session the inactive and active levers extended into the chambers. All other aspects of the FR-1 training were identical to experiment 1A.

PR self-administration training. During this phase, each infusion was followed by a timeout period of 5s, during which the pump, cue-light, and tine module were activated, and the houselight turned off. As mentioned, an important element of the PR is the fact that the response requirements increase with each infusion until the animal reaches a breakpoint, an index of an animals' motivation to work to obtain a reward. The steps on the PR schedule were calculated according as a response ratio ($[5 \times e^{(0.2 \times \text{infusion number})}] - 5$) rounded to the nearest integer (Roberts et

al., 1993). Accordingly, the number of responses required for each consecutive drug infusion was 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, etc. Thus, under the PR-1 schedule, an animal had to press once to get the first infusion, twice for the second, four times for the third, etc.; under the PR-6 schedule, for example, the animal had to press 12 times to get the first infusion, 15 times for the second, 20 times for the third, etc. In the present study, the aim was to stabilize the animals around five to six infusions per session. Therefore, following the second day on the PR-1 schedule, each animal obtaining six or more infusions had to start one step higher on the next day. For example, a rat self-administering 10 infusions on PR-1 on the third day of training was then started on the PR-2 schedule on the fourth day. This procedure was used until the animal stabilized around five to six infusions. Breakpoint attained for each animal was calculated in the following manner: an animal self-administering five infusions under a PR-6 (requiring 12 active presses to get the first infusion) was considered to have attained a breakpoint of 10 steps of the PR [(5 infusions + step 6 of the PR) – 1= 10].

PR Test. Once performance under the PR stabilized around the five to six infusions criterion, each animal was injected with ghrelin (0.0 μg, 1.5 μg, 3.0 μg/ rat/ICV in a counterbalanced manner. A minimum of two days of baseline sham injections preceded and followed each dose of ghrelin. Sham injections procedure was similar to that of the ghrelin ICV injections except no infusions were administered. The animals were taken out of their chamber and placed in a shoebox cage in the adjacent room where the infusion pump was located. The injector was inserted into the cannula and the pump was started but there was no connection

between the syringe of the microinfusion pump and the injector thus, no injection occurred. This procedure served to control for any behavioral differences that may occur due to the injection procedure.

Extinction. Following progressive ratio tests animals underwent extinction training. Extinction days followed the same procedure as the self-administration days (one 5 hr session/day). During extinction, heroin syringes were removed and thus presses on the 'active lever' previously associated with the drug no longer resulted in drug delivery. Extinction criterion consisted of two days of baseline sham injections on the PR schedule, with a breakpoint of zero. Once animals reached extinction criterion they were then subjected to a heroin-seeking reinforcement test.

Reinstatement Test. Once animals met their individual extinction criteria, animals were treated with one of three ghrelin doses (0.0 μg, 1.5 μg, or 3.0 μg/rat/ICV), 15 min before the reinstatement test under PR schedule of reinforcement set at each individual animal's PR step.

Statistical Analyses

A number of animals experienced catheter blockage or leakage preventing them from self-administering the heroin. Consequently, not all animals were subjected to all treatment conditions. As a result, only data pertaining to the animals that had received both the vehicle (0.0 μg/rat/ICV) and the high dose of ghrelin (3.0 μg/rat/ICV) *or* the vehicle (0.0 μg/rat/ICV) and the low dose of ghrelin (1.5 μg/rat/ICV) were kept for analysis. Statistical analyses were performed using SPSS Software v. 15 for Windows (SPSS Inc., Chicago, Illinois, USA). Separate repeated

measures ANOVA analyses were conducted for the effect of *ghrelin high dose* (Baseline, 0.0 µg, 3.0 µg/rat/ICV, and the effect of *ghrelin low dose* (Baseline, 0.0 µg, 1.5 µg/rat/ICV), on heroin self-administration under PR schedule for each dependent variable (breakpoint, active and inactive responses). Baseline conditions were defined as consistent breakpoints for two consecutive sham injections days before each dose. All other dependent variables' baselines were calculated by averaging the respective performance during those two stable sham days before each treatment. Statistically significant effects, or non-significant effects (n.s.) with considerable effects sizes, were followed by Fisher's LSD pair-wise comparisons. The critical cut-off point for significant results was set at $p \leq .05$.

Reinstatement Analyses. Separate mixed design ANOVAs were conducted for each dependent variable (breakpoint, active and inactive responses) recorded during the reinstatement test under PR schedule of reinforcement. The between-subjects factor was *ghrelin dose* (0.0 µg, 1.5 µg, or 3.0 µg/rat/ICV). The within-subjects factor was *day* (Baseline versus Test Day).

Results

Food intake Test. Prior to the experiment, a separate set of animals (n=5) were habituated to powdered food in the animal facility to evaluate the efficacy of ghrelin at 1 hr, 3 hr and 24 hr post-injection. Ghrelin injections resulted in dose-dependent increase in food intake where the high dose (3.0 µg/rat/ICV; M=6.0, SE=0.87) resulted in higher amount of food intake than baseline (M=4.4, SE=0.29), vehicle (0.0 µg/rat/ICV; M=4.6, SE=0.42) and low ghrelin dose (1.5 µg/rat/ICV; M=4.8, SE=0.49) conditions (See APPENDIX A, Table 4., for 3 hr and 24 hr results).

FR-1 Self-Administration Training. Animals demonstrated greater number of presses on the 'active' drug-paired lever relative to the 'inactive lever' non-drug paired lever throughout self-administration training, indicating a learned association between the active lever and drug reinforcement (Figure 8).

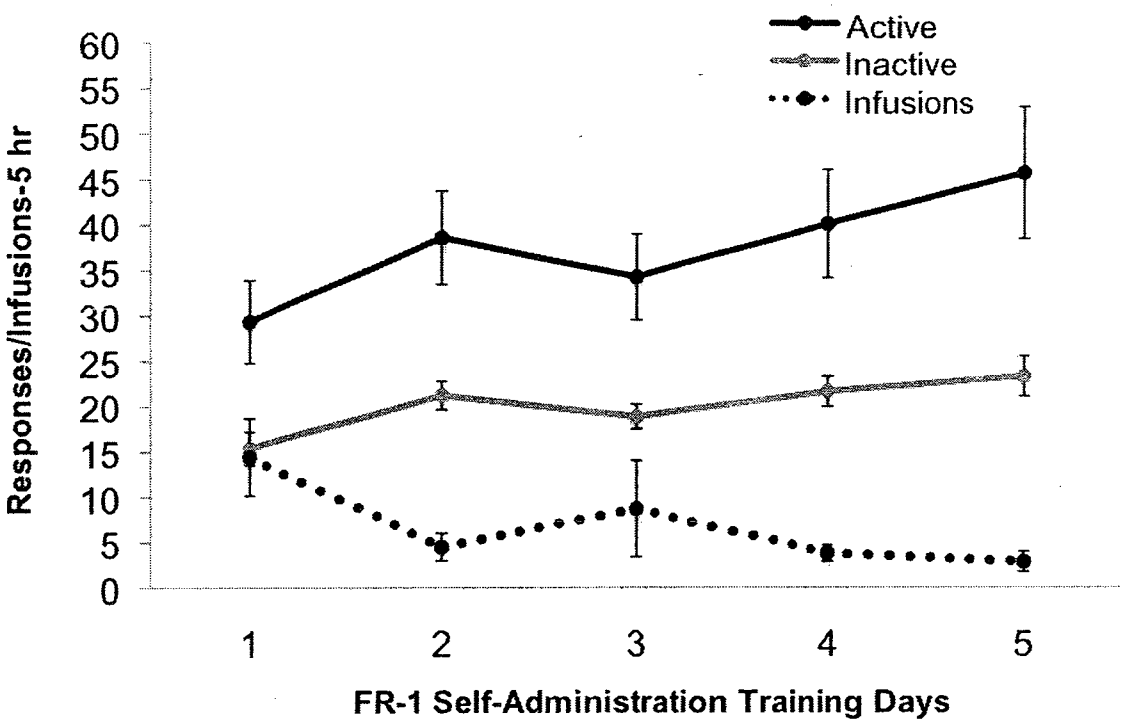

Figure 8. Last five days of heroin self-administration under FR-1 training. Mean (+/-*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.10 mg/kg/infusion) over the last 5-day training period (one 5 hr session/day, n= 14).

Progressive Ratio Test.

Ghrelin High Dose (3.0 μg; n=11). Results revealed a significant effect of ghrelin treatment on breakpoint attained with *3.0 μg* the under PR schedule of reinforcement [F (2, 20) = 4.65, p = .02]. Pairwise comparisons demonstrated that the high dose of ghrelin (M = 13.72, SE = 1.28) significantly increased the breakpoint attained (p = .038) compared to the vehicle condition (M = 11.36, SE = 1.47; p= .014) and baseline animals (M = 12.45, SE = 1.20; p= .038; Figure 9). Also, a statistically significant effect [F (2, 20) = 3.66, p = .044] for ghrelin treatment on active lever presses was found, and pairwise comparisons revealed that the number of responses with the high dose of ghrelin (M = 547.00, SE = 124.31) was significantly greater than in the vehicle condition (M = 293.91, SE = 104.59; p = .044) and baseline (M = 383.54, SE = 88.90; p = .029,) conditions. Finally, as expected, no statistically significant differences were found for inactive lever presses, all p's > .05 (Figure 10).

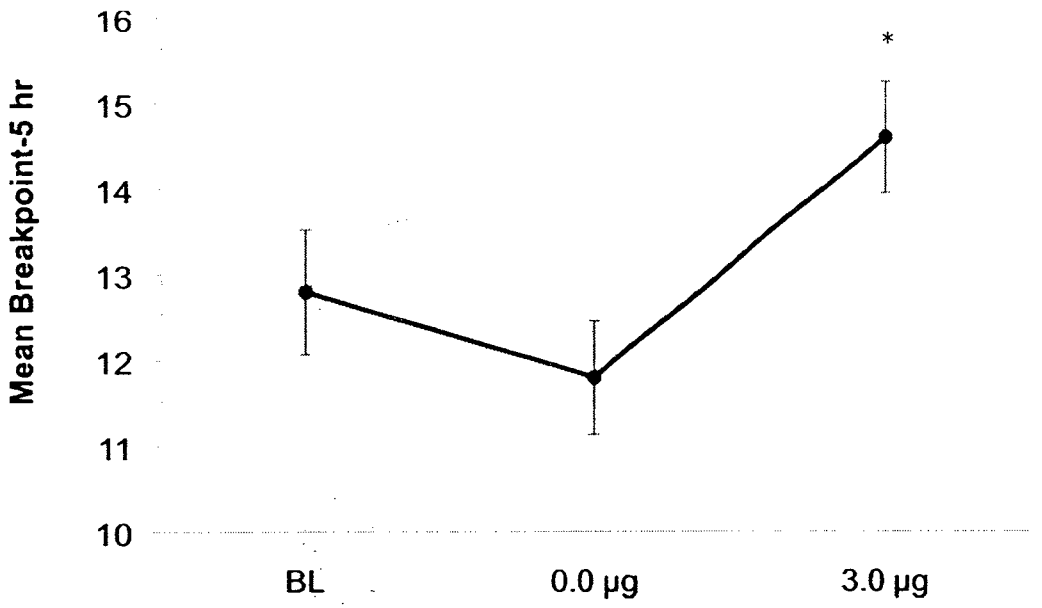

Figure 9. Mean breakpoint attained (+/- *SE*) following vehicle (0.0 µg), and ghrelin high dose (3.0 µg) treatment, 15 min before a 5 hr session under a PR schedule of reinforcement, in heroin (0.1 mg/kg/infusion) self-administering rats, $n = 11$. Baseline (BL) conditions were defined as the average of breakpoints on the 2 days before each dose treatment. * $p < .05$ when compared to baseline and vehicle treated animals.

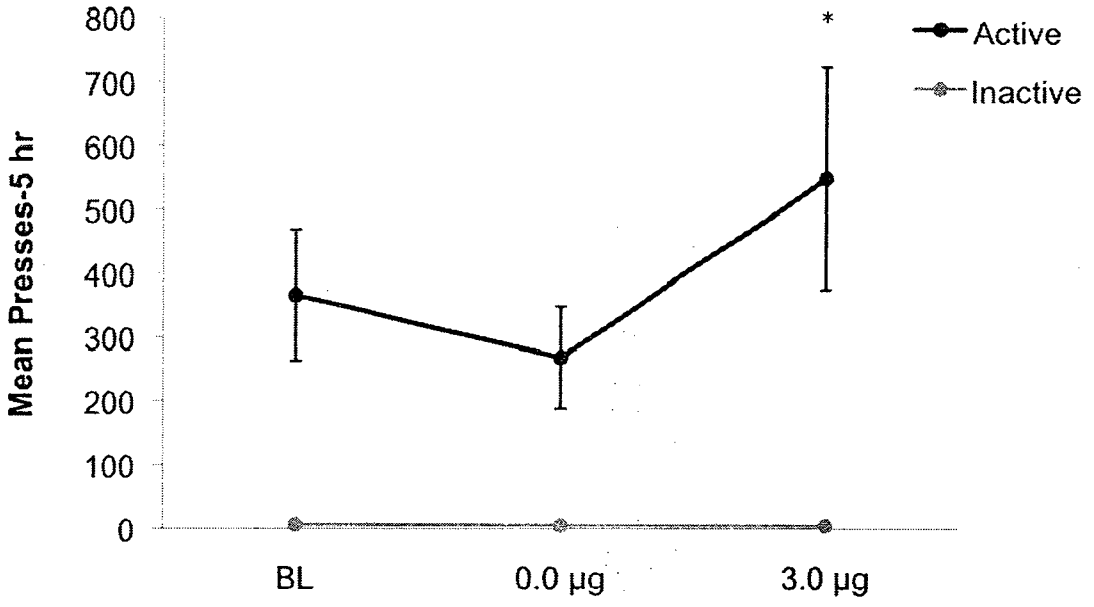

Figure 10. Mean (+/- *SE*) number of active and inactive lever presses performed following vehicle (0.0 µg), and ghrelin high dose (3.0 µg) treatment, 15 min before a 5 hr session under a PR schedule of reinforcement, in heroin (0.1 mg/kg/infusion) self-administering rats, n = 11. Baseline (BL) conditions were defined as the average of lever presses on the 2 days before each dose treatment. * $p < .05$ when compared to baseline and vehicle treated animals.

Ghrelin Low Dose (1.5 μg; n=4). Results revealed no statistically significant effect for ghrelin treatment on breakpoints under PR schedule of reinforcement [F (2, 6) = 2.56, p = .16]. Since we obtained a moderate effect size (*Partial* η^2 = .46) and considering the fact that our animal number was low (n = 4), pairwise comparisons were performed which revealed a statistically significant difference (p = .01) in breakpoint attained between baseline (M = 10.56, SE = .77) and *1.5 μg* of ghrelin (M = 11.75, SE = .85) conditions (Figure 11). No statistically significant effect for treatment with *1.5 μg* of ghrelin on active lever presses was found. However, since the effect size obtained was moderate (*Partial* η^2 = .54) pairwise comparisons were performed which revealed a statistically significant difference (p = .02) in active lever presses between baseline (M = 205.12, SE = 54.17) and *1.5 μg* of ghrelin (M = 299.00, SE = 69.80) conditions. Finally, as expected, no statistically significant differences were found for inactive lever presses, all p's > .05 (Figure 12).

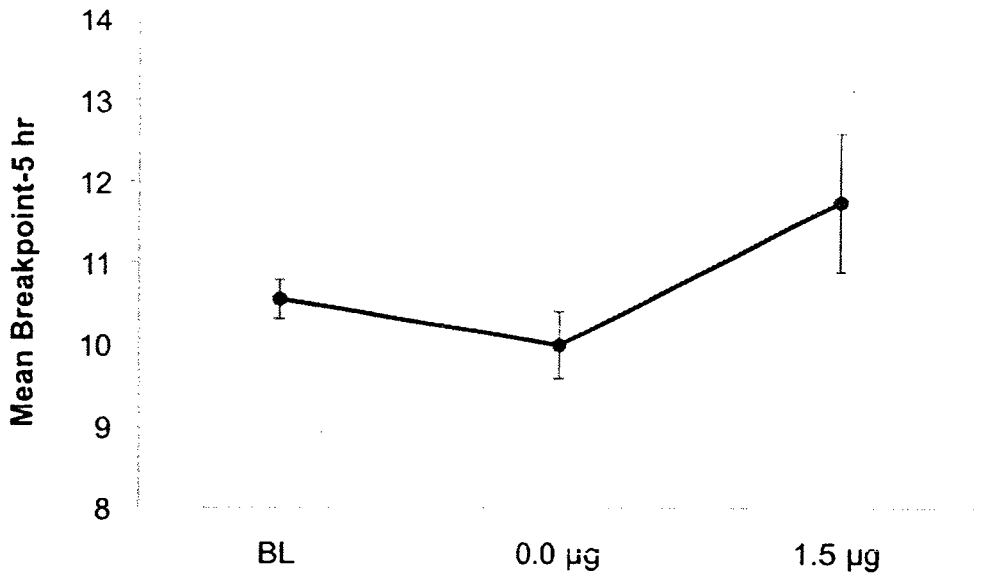

Figure 11. Mean breakpoint attained (+/- *SE*) following vehicle (0.0 µg), and ghrelin low dose (1.5 µg) treatment, 15 min before a 5 hr session under a PR schedule of reinforcement, in heroin (0.1 mg/kg/infusion) self-administering rats. Baseline (BL) conditions were defined as the average of breakpoints on the 2 days before each dose $n = 4$.

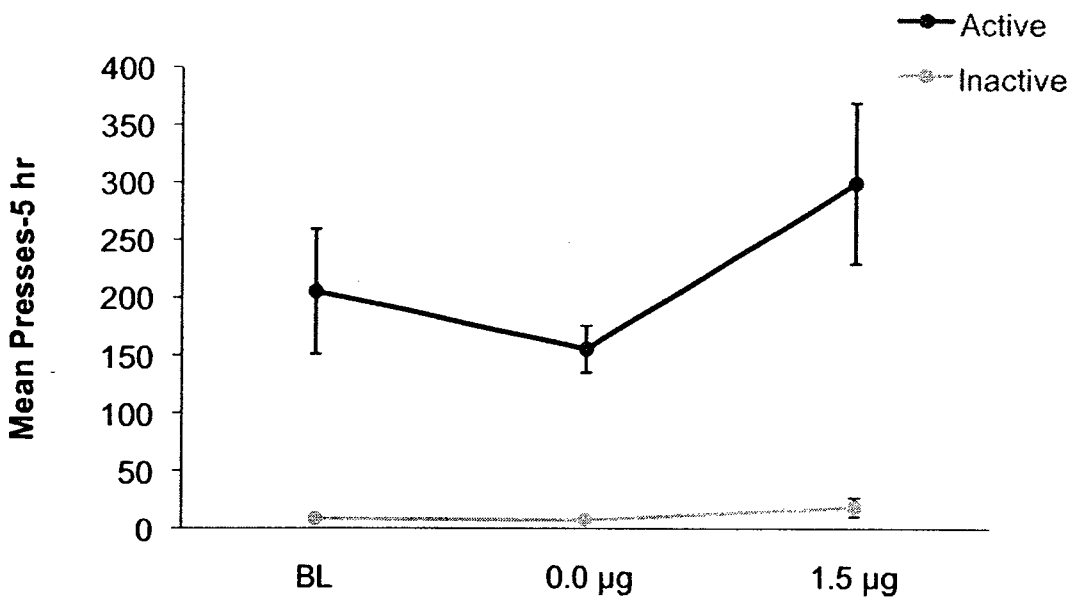

Figure 12. Mean (+/- *SE*) number of active and inactive lever presses performed following vehicle (0.0 µg), and ghrelin low dose (1.5 µg) treatment, 15 min before a 5 hr session under a PR schedule of reinforcement, in heroin (0.1 mg/kg/infusion) self-administering rats. Baseline (BL) conditions were defined as the average of breakpoints on the 2 days before each dose; $n = 4$.

Reinstatement Test. Following self-administration PR test and having met the extinction criterion, animals were subjected to reinstatement tests (n=11). These rats experienced 15-32 days of heroin self-administration training under PR schedule. Results demonstrated no significant interaction between *day* (baseline versus test day) and *ghrelin dose* (0.0, 1.5 or 3.0 μg) for breakpoints [$F(4, 40)=2.73$, $p=.124$]. The effect of *day* on the animals' breakpoint approached significance [$F(2, 8) = 4.84$, $p = .059$]. No significant differences were found for the effect of *ghrelin dose* on the animals' breakpoint [$F(2, 8)=2.73$, $p=.124$]. Since the effect size for ghrelin dose obtained was moderate (*Partial* $\eta^2 = .40$) pairwise comparisons were performed which revealed an effect aproaching statistical significance ($p = .053$) where *ghrelin high dose* (n=6; M= 4, SE=1.02) resulted in an increase in breakpoint as compared to vehicle treated animals (n=3; M=0, SE=1.443) regardless of test day (Figure 13).

Results demonstrated a non-significant interaction between *day* and *ghrelin dose* [$F(2, 8) = 0.83$, $p = .470$] for the active lever presses. No significant main effects for *day* [$F(2, 8) = 2.80$, $p = .133$] and for *ghrelin dose* [$F(2, 8) = 1.05$, $p = .392$] were found. However, it is important to note that although non-significant, the ghrelin low (M=99.5, SE=74.5) and high (M=99.33, SE=48.13) doses displayed similar patterns of greater number of active presses, as compared to vehicle treated animals (M=5.66, SE=3.6; Figure 14). As expected, ghrelin treatment had no statistically significance effect on the inactive lever presses all p's > .05 (Figure 15).

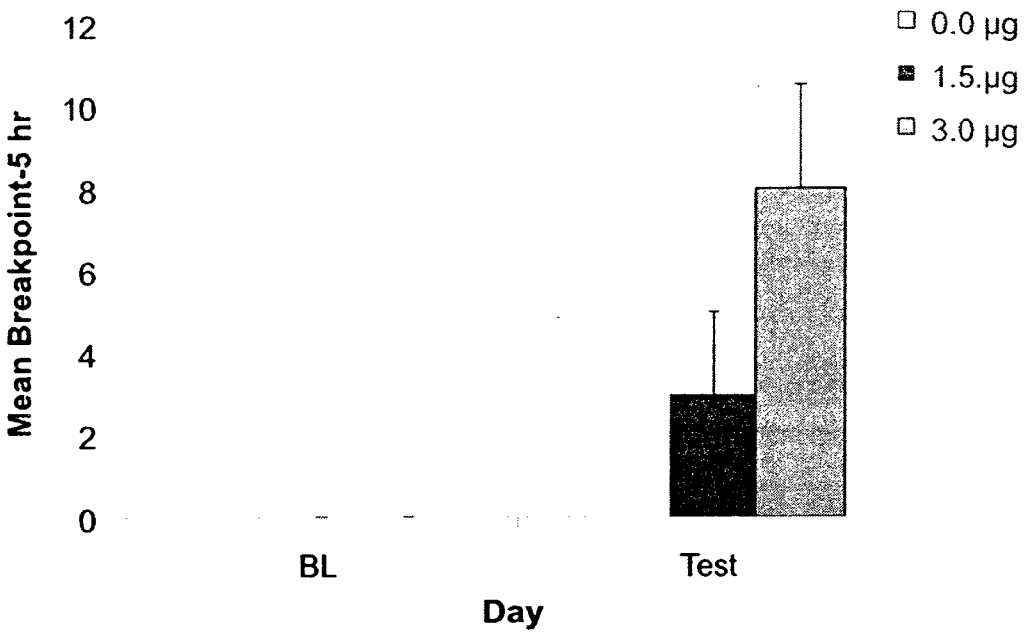

Figure 13. The effect of ghrelin on reinstatement of heroin-seeking under a PR schedule of reinforcement. Data presented are mean (+*SE*) breakpoints following ghrelin infusion (0.0, 1.5, or 3.0 µg) 15 min before the session. Baseline (BL) condition represents mean responding on the last extinction day prior to each test session.

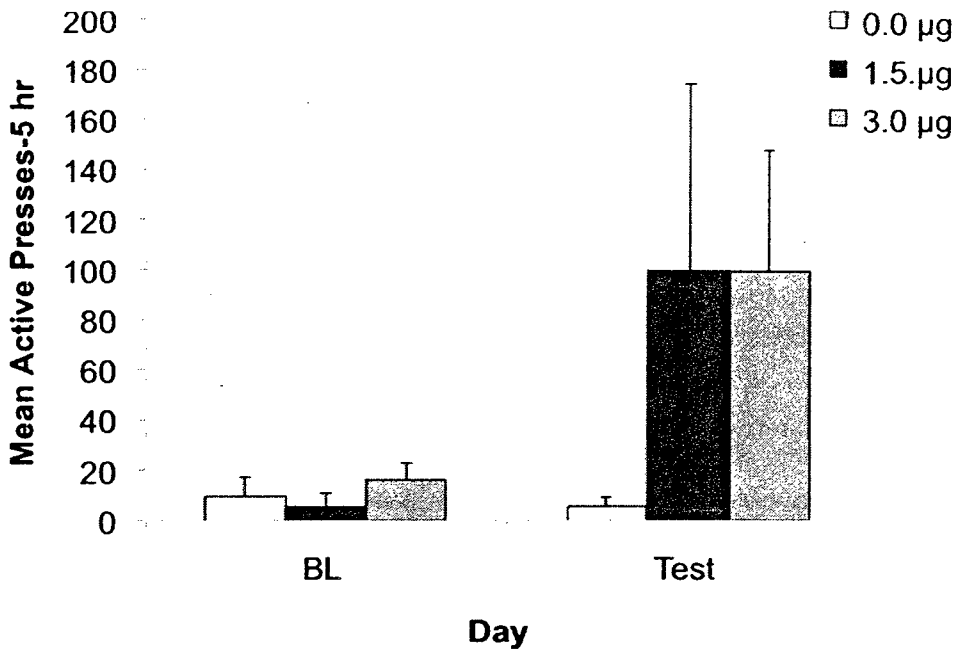

Figure 14. The effect of ghrelin on reinstatement of heroin-seeking under a PR schedule of reinforcement. Data presented are mean (+*SE*) number of active lever responses following ghrelin infusion (0.0, 1.5, or 3.0 µg) 15 min before the session. Baseline condition (BL) represents mean responding on the last extinction day prior to each test session.

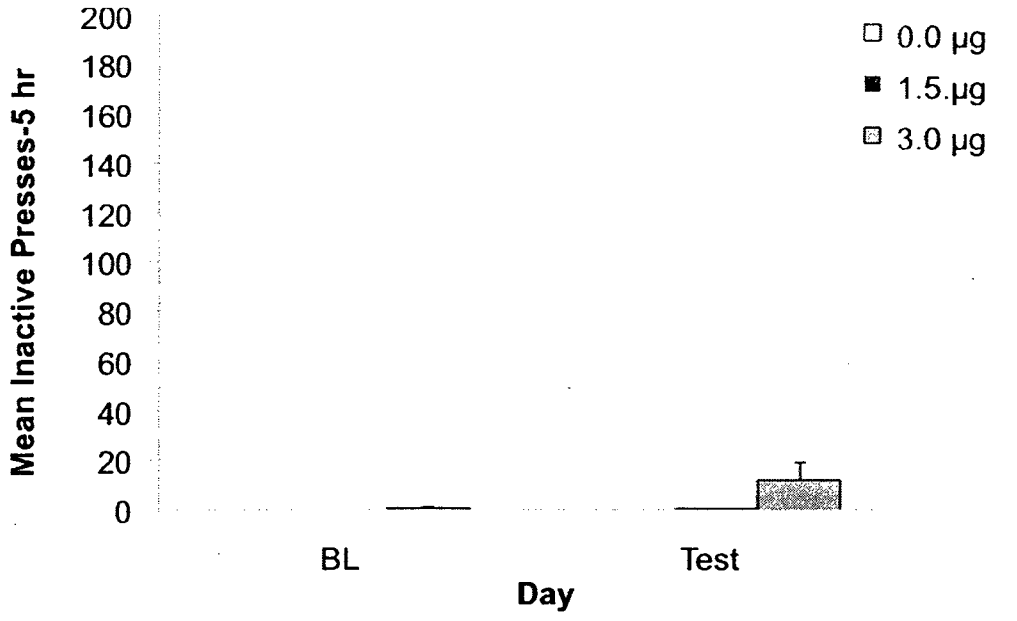

Figure 15. The effect of ghrelin on reinstatement of heroin-seeking under a PR schedule of reinforcement. Data presented are mean (+*SE*) number of inactive lever responses following ghrelin infusion (0.0, 1.5, or 3.0 µg) 15 min before the session. Baseline condition (BL) represents mean responding on the last extinction day prior to each test session.

Summary

Experiment 3 demonstrated that central ghrelin administration could augment the motivational properties of heroin, as demonstrated by increased breakpoints under a PR schedule of reinforcement. Moreover, results showed a trend for ghrelin-induced reinstatement of heroin-seeking under extinction conditions on a PR schedule. These results complement previous findings on the effects of NPY on heroin self-administration and reinstatement of heroin-seeking (Maric et al., 2008), and the results presented here with the NPY Y5-receptor antagonist, Lu AA333810. It is suggested that ghrelin augments reward-related behavior, and thus it is possible that activation of its receptors, which enhance the release of NPY, is critical for the expression of FD-induced reinstatement of heroin seeking. Therefore, we examined the effect of ghrelin antagonism on on-going heroin self-administration and FD-induced reinstatement.

EXPERIMENT 4: Effects of ghrelin antagonism on heroin self-administration and food deprivation-induced reinstatement of heroin-seeking

Method

Subjects

Subjects were 20 Long Evans male rats (Charles River, St. Constant, QC) that weighed 275-300g at the beginning of the experiment. Prior to surgery, the rats were housed in pairs for 1 week, in the animal facility at Concordia University, under a reverse light/dark cycle (Lights off at 9:30AM, on at 9:30PM). *Ad libitum* access to

water and food was available, except for the 21 hr food deprivation condition. Body weights were measured daily.

Surgery

Surgical procedures were identical to methods described in experiment 1A. Briefly, animals were implanted with intravenous silastic catheters into the right jugular vein to allow for drug-self-administration, and a unilateral 23-gauge guide cannula aimed 2 mm above the right lateral ventricle (AP - 0.5, ML +1.4, DV -2.0, relative to bregma) was implanted to allow for the ICV injections of ghrelin antagonist.

Apparatus

Throughout the experiment, the animals were individually housed in one of 10 identical operant chambers (29.0 cm x 29.0 cm x 25.5 cm; Coulbourn Instruments, Allentown, PA, USA) as previously described.

Drugs

Heroin (National Institute for Drug Abuse, Baltimore, MD) was dissolved in sterile distilled water (5mg/ml), which was then further diluted with 0.9% saline solution according to body weight to a dose of 0.1 mg/kg/infusion. Ghrelin receptor antagonist ([D-Lys-3]-GHRP-6, H-His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH2; PEPTIDES International, KY) was dissolved to a concentration of 5 μg/μl in sterile saline solution and delivered at a volume of 4 μl over 4 min to reach a dose of 0.0, or 20.0 μg/rat, The dose selected has been shown to reliably inhibit feeding behavior in animals (Fujino et al., 2003).

Procedure

Self-Administration Training. Following one day of habituation and auto shaping, self-administration training was conducted over 10-12 days, consisting of three 3 hr sessions per day under FR-1 schedule of reinforcement. All aspects of self-administration training were identical as those described in experiment 1A.

Self-Administration Test. Half of the rats (n = 10), were subjected to a minimum of two baseline sham injections during the last two days of self-administration training. Once rats reached a stable drug infusion criterion (less than 10% variability over two consecutive days), they received injections of the ghrelin receptor antagonist (20.0 μg/rat ICV) and vehicle (saline, 4 μl/rat/ICV) in a counterbalanced order, 15 min before the first session of the test day. The injector extended 2 mm below the implanted guide cannula and was kept in place for another 60 s after the injection to allow for complete diffusion of the ghrelin antagonist.

Extinction. Following self-administration training (and tests in some animals), animals underwent extinction training. Extinction days followed the same procedure as the training days, but consisted of only one 3 hr session/day, with the exception of the first day of extinction, which consisted of a three 3 hr sessions/day. During extinction, heroin syringes were removed and thus presses on the 'active lever' previously associated with the drug no longer resulted in drug delivery.

Extinction training continued for a minimum of 4 days and until animals reached an extinction criterion of 20 or less active presses per 3 hr session.

Reinstatement Test. Once animals met the extinction criterion, they were exposed to two 3-hr reinstatement test sessions, which were preceded by either 21 hr FD (food hoppers removed) or 21 hr of unlimited access to food, in a counterbalanced order. Rats were injected with one of two the ghrelin receptor antagonist doses (0.0, or 20.0 μg/rat, ICV) 15 min before each test. Approximately 30 minutes following the completion of the food-deprivation tests, food hoppers were returned. Each test session was followed by and preceded by a minimum of two baseline days.

Statistical Analyses.

Self-Administration Test. Self-administration data were analyzed using separate repeated–measures ANOVA for each dependent variable (infusions, active and inactive presses, recorded over one 3-hr session) with one within-subjects factor of *ghrelin antagonist treatment* (baseline, 0.0 and 20.0 μg/ rat/ICV). Baseline conditions were calculated by averaging the last two days of self-administration training that reached testing criteria preceding the reinstatement tests.

Reinstatement Test. Reinstatement data were analyzed by using a mixed design ANOVA with a within-subjects factor of *condition* (baseline, 21 hr food deprivation and *ad libitum*), and a between subject factor of *ghrelin antagonist dose* (0.0 or 20.0 μg/rat/ICV). Baseline condition was calculated by averaging the last of day of extinction before each of the reinstatement tests. Significant results were followed up with Fisher's LSD post-hoc comparisons. The critical cut-off point for significant results was set at $p \leq 0.05$.

Results

Food intake Test. Prior to the experiment, a separate set of animals (n=2) were habituated to powdered food in the animal facility to evaluate the efficacy of the ghrelin antagonist at 1 hr, 3 hr and 24 hr post-injection. Treatment with ghrelin antagonist (20.0 μg/rat/ICV) reduced food intake (M=0.0, SD=0.0) compared to baseline (M=2.34, SE=0.81) and vehicle (M=2.3, SE=0.25) conditions at 1 hr post-injection (See APPENDIX A, Table 5., for 3 hr and 24 hr results).

Self-administration Training. Animals demonstrated greater number of presses on the 'active' drug-paired lever relative to the 'inactive lever' non-drug paired lever throughout self-administration training, indicating a learned association between the active lever and drug reinforcement (Figure 16).

Figure 16. Heroin self-administration training. Mean (+/-*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.10 mg/kg/infusion) over the 10-day training period (three 3h sessions, n= 19).

Self-Administration Test. Following self-administration training and upon reaching a steady infusion rate, half of the animals (n = 10) were subjected to tests with two ghrelin antagonist doses (0.0 or 20.0 μg/rat/ICV). Data from one animal were removed from the self-administration analysis due to catheter leakage before infusion rate stabilized. The final analyses were conducted using data from nine rats. No significant effect for *ghrelin antagonist treatment* on heroin infusions was obtained, [$F(2, 16) = 0.114$, $p = .893$; Figure 17]. No significant effects were found for the ghrelin antagonist treatment on active lever presses [$F(2, 16) = 1.51$, $p = 0.25$; Figure 18]. In addition, no significant effects were found for the inactive lever presses, all p's > .05.

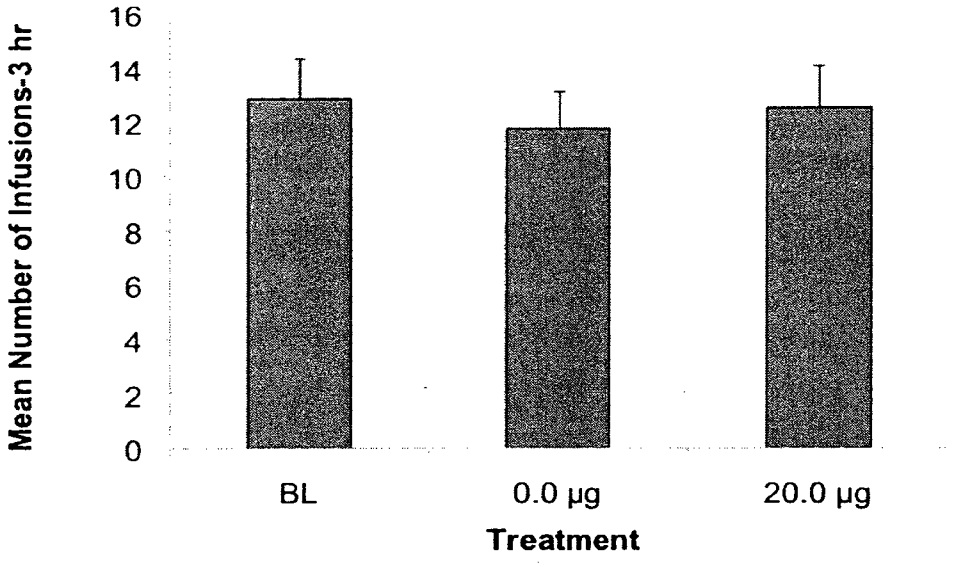

Figure 17. The effect of treatment with ghrelin receptor antagonist during on-going heroin self-administration (0.1 mg/kg/infusion) under FR-1 schedule of reinforcement. Data presented are mean (+*SE*) number of infusions obtained over one 3-hr session, following ghrelin receptor antagonist injection (0.0 and 20.0 µg/rat/ICV, $n = 9$) 15 min before the first session of the test day. Baseline condition (BL) represents the average number of infusions on the last two days of training prior to each test session.

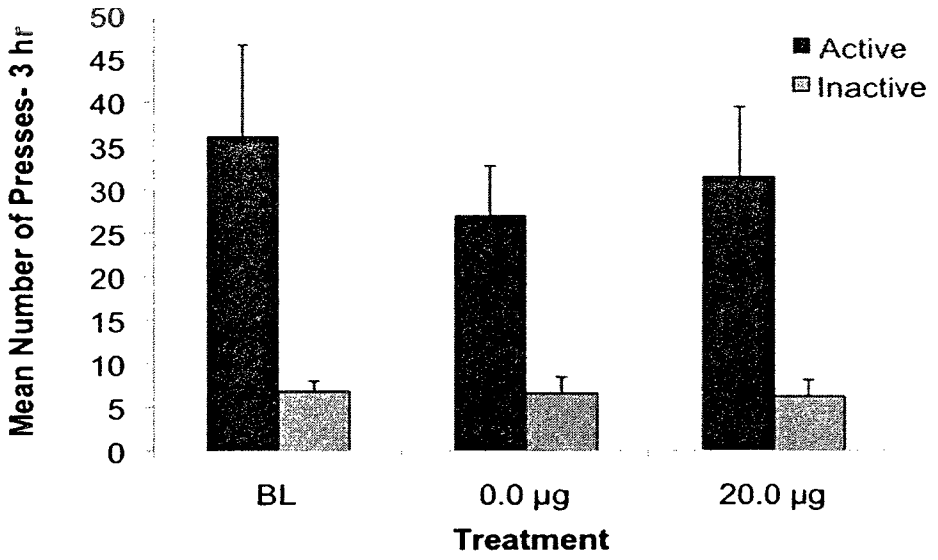

Figure 18. The effect of treatment with ghrelin receptor antagonist during on-going heroin self-administration (0.1 mg/kg/infusion) under FR-1 schedule of reinforcement. Data presented are mean (+*SE*) active and inactive lever responses obtained over one 3-hr session following ghrelin receptor antagonist injection (0.0 or 20.0 µg/rat/ICV, n =9) 15 min before the test sessions. Baseline condition (BL) represents the average number of responses on the last two days of training prior to each test session.

Reinstatement Test. Following extinction, animals were subjected to one of two ghrelin antagonist doses (0.0 or 20.0 µg/rat/ICV). One rat was removed from the analysis because of an extreme response during the food deprivation vehicle (0.0 µg/rat/ICV) condition (141 active lever responses compared to a mean of 23.22). Final analyses included data from 19 rats in two treatment groups of *ghrelin antagonist dose* 0.0 µg (n=9) and 20.0 µg (n=10). Results demonstrated a non-significant interaction [$F(2, 34)=1.75$, $p=.188$] between *condition* and *ghrelin antagonist dose* for active lever presses. A significant main effect for *condition* on active lever presses, [$F(2, 34)=6.43$, p=.004] was found. Pairwise comparison demonstrated that the number of active lever responses in the food deprivation condition (M=23.73, SE=4.02) was higher compared to baseline (M=11.84, SE=0.67) and *ad libitum* (M=16.46, SE=3.33) conditions. There was no significant main effect of *ghrelin antagonist dose*, [$F(1,17)=1.159$, $p=.297$]. Although the *condition* x *ghrelin antagonist dose* was not statistically significant, a notable difference between the vehicle (0.0 µg; M=10.33, SE=3.05) and ghrelin antagonist (20.0µg; M=22.60, SE=5.68) in the *ad libitum* condition was observed (Figure 19). In addition, no significant effects were found for the inactive lever presses, all p's > .05.

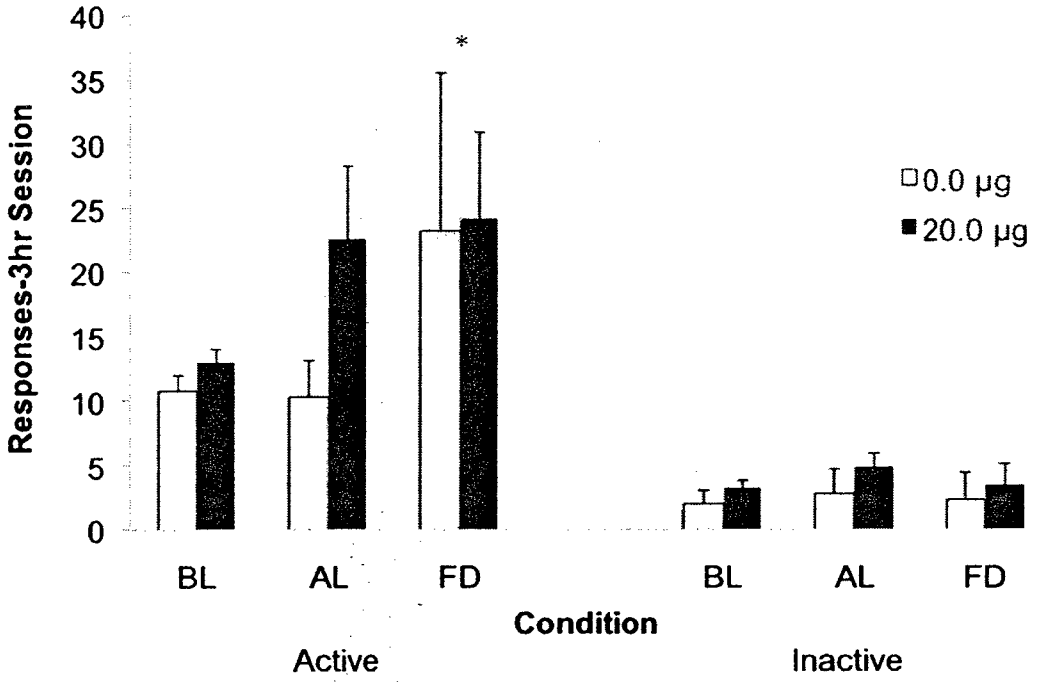

Figure 19. The effect of treatment with ghrelin receptor antagonist on 21 hr FD-induced reinstatement of heroin seeking. Data presented are mean (+*SE*) active (left) and inactive (right) lever responding during baseline (BL), and following ghrelin receptor antagonist injection (0.0 or 20.0 µg/rat/ICV), 15 min before the session, on the 21 hr FD, or *ad libitum* (AL) test days. Baseline condition (BL) represents mean responding on the last day of extinction prior to each test session. * Significant difference ($p < 0.05$) between the FD condition and the BL and AL conditions.

Summary

The results in experiment 4 were somewhat unexpected, as we have shown that ghrelin can increase the motivation for heroin self-administration (experiment 3), and others have shown a sensitizing effect for ghrelin on cocaine-induced hyperactivity (Wellman et al., 2005), and CPP (Davis et al., 2007). However, treatment with a dose of ghrelin antagonist known to effectively suppress food intake, had no effect on heroin self-administration or FD-induced reinstatement of extinguished heroin seeking behavior, suggesting that ghrelin has a minor, non-critical, role in mediating the rewarding effects of drugs and the augmentation of drug-seeking behavior by food deprivation.

General Discussion

In humans, drug abuse commonly coexists with eating disorders (Piran & Gadalla, 2006). The ratio of substance abuse disorders among those with eating disorders is approximately 25.7%, eight times higher than the general population. In addition, the ratio of eating disorders rises to about 16.3% among those with substance abuse disorders, which is five times greater than general population (Blinder, Blinder & Sanathara, 1998). Given this high level of co-morbidity, it has been suggested that these disorders share a common neurobiological underpinning, where a dysregulation or alteration in an underlying neuronal system occurs. This notion is supported by reports in the literature over the last three decades indicating that the rewarding effects of drugs of abuse activate the same system that evolutionarily developed to process rewarding and reinforcing environmental stimuli (Volkow & Wise, 2005). It is thus not surprising that many have suggested that the hormones which mediate homeostatic feeding and food intake can modulate and be modulated by drugs of abuse (DiLeone, Georgescu & Nestler, 2003; Carr, 2002).

Peripheral and central peptidergic signaling which regulate homeostatic feeding, are elicited in response to internal and external conditions of an organism. For example, upon periods of food restriction, circulating ghrelin is released in the periphery, signaling to NPY/AgRP neurons to enhance their release. Evidence has shown that ghrelin, as well as leptin, act on this NPY/AgRP circuit in order to regulate feeding behavior (Cowley et al., 2003) which suggests that peripheral and

central orexigenic signals work in tandem to mediate food intake during periods of energy deficit. Moreover, the effect of these orexigenic signals extends beyond the control of feeding to restore energy balance, and includes a direct effect on the hedonic value of food reward (Saper, Chou & Elmquist, 2002). Since the central activity of these hormones is important for the regulation of food reward, it is possible that their role in drug-related behavior, namely food-deprivation-induced reinstatement of drug seeking, is critical. The goal of the present thesis was to demonstrate that orexigenic hormones known to be involved in homeostatic regulation and feeding behavior (i.e. NPY, ghrelin) have modulatory roles on drug-related phenomena. It was proposed that by manipulating orexigenic activity, a dysregulation in the reward system would occur and thus alter drug-related behavior. The current thesis provided evidence for a role of these orexigenic hormones in drug-related behavior, where NPY Y5, but not the Y1-receptor, is necessary for food-deprivation induced reinstatement of heroin seeking-behavior. This effect was shown to be independent of central ghrelin transmission as antagonism of its receptors failed to modulate food-deprivation induced reinstatement, although activation of central ghrelin receptors augmented the motivation for drug taking.

The role of NPY in acute food deprivation-induced reinstatement of heroin seeking

We have previously found that ICV injections of NPY, at doses known to stimulate feeding, augmented cocaine self-administration and cocaine-induced locomotor activity, and induced reinstatement of previously extinguished heroin-seeking behavior in free-feeding animals (Maric et al., 2008; 2009). These findings imply that NPY mediates both reinforcing and conditioned properties of drugs of

abuse. We have suggested that injections of NPY had the ability to activate or to "mimic" a state of food-deprivation. Thus, it was concluded that NPY is involved in FD-induced reinstatement perhaps via a homeostatic mechanism mediated by "hunger"-like state, or by direct augmentation of the reinforcing properties of conditioned drug reward. It was further speculated that NPY transmission might be necessary for the expression of FD-induced reinstatement. In order to investigate this issue, the current thesis hypothesized that targeting NPY Y1- and Y5-receptors which are involved in mediating NPY's orexigenic activity, would attenuate food-deprivation induced reinstatement of heroin-seeking, albeit through different pathways due to their specific involvement in multiple neurobiological processes.

Involvement of the NPY Y1-receptor in acute food deprivation-induced reinstatement of heroin seeking behavior

The distribution of NPY Y1-receptors is widespread. Although NPY receptors are found within peripheral tissues including the heart, kidneys, and gastrointestinal tract, most Y1 receptors are found within the brain in hypothalamic areas at sites involved in the regulation of energy balance such as the ARC, PVN, and lateral hypothalamus (LH) (Parker & Herzog, 1999) and are also found in the basolateral amygdala (BLA) and nucleus accumbens (NAc; Pickel, Beck-Sickinger, Chan & Wieland, 1998). Hypothalamic NPY Y1-receptors are thought to mediate the orexigenic properties of NPY, where blocking of these receptors reduce feeding behavior in free-feeding and diet-induced obese animals (Kanatani et al., 2001). Thus, targeting Y1 receptors was important, as it was hypothesized that states of food deprivation mediated by the Y1 receptor activity would in turn modulate FD-induced

reinstatement of drug-seeking behavior. Experiment 1A demonstrated that ICV administration of the NPY Y1-receptor antagonist BIBO 3304 did not attenuate drug-seeking in food deprived animals. Results indicated that under food-deprived conditions, robust heroin-seeking behavior was found, as demonstrated by increases in responding on the active lever, previously associated with drug delivery, compared to baseline and *ad libitum* conditions, regardless of NPY Y1-receptor antagonist treatment. These results are in agreement with previous findings demonstrating that acute food deprivation causes reinstatement of drug-seeking (Shalev et al., 2000). However, the absence of attenuation via Y1-receptor antagonism is somewhat unexpected in light of our previous findings (Maric et al., 2008; 2009), showing that NPY caused restoration of extinguished heroin-seeking behavior in animals with *ad libitum* food access. These surprising findings cannot, however, be regarded as conclusive evidence that FD-induced increases in NPY transmission have no role in FD-induced reinstatement of drug seeking behavior. Instead, it seems that NPY Y1-receptors do not play a critical role in food deprivation-induced reinstatement of drug seeking.

Moreover, it appeared that NPY Y1-receptor antagonist augmented, rather than attenuated, the reinstatement effect, exclusively in the food-deprivation condition. Although statistical significance was not established and such assumptions are not usually recommended, an impressive effect size found within the food-deprivation condition suggests that a synergistic effect between the food deprivation condition and Y1-receptor antagonist treatment might be present and thus merits consideration. An augmentation in FD-induced reinstatement could be explained by

the anxiogenic properties of Y1-receptor antagonists. It has been shown that NPY transmission reduces anxiety related phenomena (Heilig, Soderpalm, Engel, & Widerlove, 1989), an effect found to be mediated by the Y1 receptor, as treatment with the Y1-receptor antagonist, BIBP 3226 induced conditioned place-avoidance (Kask, Kivastik, Rago, & Harro, 1999). Similar findings have been reported using BIBO 3304, a more specific, high affinity Y1-receptor antagonist (Wieland et al., 1998). ICV Injections of BIBO 3304 in a novel open field test, resulted in an increased defecation, a measure of anxiety, that occurred without affecting locomotion (Kask & Harro, 2000).

Based on the suggested role of the Y1-receptor activation in the anxiolytic properties of NPY, we propose that the effects observed within the food deprivation condition in experiment 1A might have been a result of an increased anxiogenic experience associated with the acute food deprivation, due to the treatment with the Y1-receptor antagonist. This hypothesis was supported by our complimentary findings with the elevated-plus maze test in experiment 1B. Animals treated with the NPY Y1-receptor antagonist, BIBO 3304, under food-deprived conditions, demonstrated an increase in time spent on the closed arms and a reduction in time spent on the open arms of the elevated plus maze. These results complement those previously found with antisense inhibition of the Y1 receptor expression, which increased anxiety in the plus-maze test (Wahlestedt, Pich, Koob, & Heilig, 1993). Together, the findings in experiments 1A and 1B support the idea of a synergistic effect between the food deprivation stress condition and Y1-receptor antagonist-induced anxiety. Further studies are needed in order to evaluate whether these effects

are specific to food deprivation or generalize to other stressors known to induce reinstatement of extinguished drug seeking.

In light of previous findings it is postulated that the present results might represent an effect of Y1-receptor antagonism in areas that are not involved in its homeostatic role. Heilig and colleagues have demonstrated that an important anatomical substrate responsible for the anxiolytic effects of NPY is the amygdala, where intra-amygdala NPY injections produce anxiolytic actions without influencing feeding behavior, effects which were blocked by ICV administration of Y1-receptor antisense (Heilig et al., 1993; Heilig, 1995). Moreover, injection of the Y1-receptor antagonist BIBP 3226 administered intra-periaqueductal gray (PAG) but not the intra-PVN produced anxiogenic-like effects (Kask, Rago, & Harro, 1998b).

The potential involvement of NPY Y1-receptors in the amygdala in the augmentation of FD-induced reinstatement is supported by the demonstration that stress-induced relapse is independent of hypothalamic-pituitary adrenal axis (HPA) activity (Shaham et al., 1997; Shalev, Finnie, Quinn, Tobin, & Wahi, 2006). Instead, corticotropin-releasing factor (CRF) transmission in extra-hypothalamic sites seems to be critical for footshock and FD-induced reinstatement of drug seeking (Erb, Shaham & Stewart, 1998; Erb, Salmaso, Rodaros & Stewart, 2001; Shalev et al., 2006). Importantly, it has been suggested that NPY and CRF are co-localized within brain nuclei associated with anxiety responses and have opposing effects on the stress system; where CRF release within the BLA is followed by NPY release, which dampens the CRF-induced stress response, acting as a compensation mechanism (Heilig, Koob, Ekman & Britton, 1994; Hastings, McClure-Sharp & Morris, 2001).

Erb et al. (2001) have demonstrated that in fact it is the bed nucleus of the stria terminalis (BNST), receiving gamma-Aminobutyric acid (GABA)-ergic and CRF input from the central nucleus of the amygdala (CeA) that is critical for footshock-induced reinstatement of extinguished cocaine seeking. Interestingly, it appears that NPY and CRF interactions also mediate GABA-ergic transmission within the BNST (Kash & Winder, 2006). These findings could indicate that reinstatement to drug seeking by non-specific NPY receptor activation, as we have recently found (Maric et al., 2008), might have activated receptor sites all over the brain, including hypothalamic sites, producing a hunger-like state. In contrast, specific Y1- receptor inactivation might have resulted in an anxiety-like state, mediated through extra-hypothalamic sites such as the amygdala, leading to an augmentation of the food deprivation effect. However, this interpretation cannot explain why we did not find BIBO 3304 -induced reinstatement effect in sated rats. One way to explain the effect found exclusively in the food-deprivation condition is through the blockade of the anxiolytic effects of the compensatory stress-induced phasic release of NPY in critical brain areas (e.g., amygdala, septum; Heilig, 1994) by BIBO 3304, which would result in an augmented response only in animals exposed to the FD stress. The anxiogenic effects of BIBO 3304, when administered to sated rats, might not have been severe enough to induce reinstatement of drug seeking. Finally, the conclusion presented above regarding the involvement of particular brain areas in our findings should be reviewed with caution since the present thesis did not involve site-specific injections of the Y1-receptor antagonist.

In conclusion, results from experiment 1A demonstrated that NPY Y1-receptor is not critically involved in FD-induced reinstatement of previously extinguished drug seeking. Moreover, it appears that NPY Y1-receptor antagonism might augment the effects of food-deprivation by inducing anxiety-like states. Thus, it is possible that NPY-induced reinstatement of drug-seeking behavior previously observed, could be mediated by another receptor subtype.

Involvement of the NPY Y5-receptor in acute food deprivation-induced reinstatement of heroin seeking behavior

The moderate effects found for NPY-induced reinstatement of drug seeking (Maric et al., 2008) might be due to the abundance of NPY receptors in the central nervous system, which upon activation, often have opposing effects (Pedrazzini, Pralong, & Grouzman, 2003; Kask et al., 2002). NPY Y5-receptor is abundantly expressed in hypothalamic areas known to regulate food intake such as the PVN, ARC, and in limbic regions (Wolak et al., 2003; Morin & Gehlert, 2006). Although the NPY Y1-receptor is critical in the regulation of food intake, activation of the Y5 receptors has also been shown to stimulate feeding (Mullins et al., 2001; Marsh, Hollopeter, Kafer, & Palmiter, 1998). Results from the current study demonstrated that unlike Y1, NPY Y5-receptors are critically involved in FD-induced reinstatement of heroin seeking.

Experiment 2A demonstrated that ICV administration of the Y5-receptor antagonist, L-152 802, had no effect on FD-induced reinstatement of extinguished

responding for heroin. Although these initial results implied that Y5-receptors were not involved in FD-induced reinstatement, a novel, selective antagonist Lu AA33810 was recently demonstrated to have greater affinity for the Y5 receptor (Walker et al., 2009), and is thus considered a superior candidate for use in our procedure. Contrary to experiment 2A, experiment 2B demonstrated that treatment with Lu AA33810 resulted in a robust attenuation of FD-induced reinstatement of extinguished heroin-seeking. These effects were not due to general, non-specific effects of the antagonist, as the antagonist had no effect on the inactive lever responding. Results from experiment 2A are not consistent with studies that have shown that L-152-804 suppresses reward-related behavior. For example, it has been shown the oral delivery of L-152-804 attenuated alcohol-self-administration in alcohol preferring rats (Schroeder, Overstreet & Hodge, 2005). Moreover, unpublished data from Woldbye's group has demonstrated that L-152-804 attenuated cocaine self-administration, and relapse to cocaine-seeking in a CPP model (Sorensen, Fink-Jensen, Wortwein, & Woldbye, 2008). The discrepancies between experiment 2A and 2B using different Y5-antagonists, and with previous reports using L-152-804, are important to discuss before interpreting the role of Y5 receptors in FD-induced reinstatement. First, and perhaps most importantly to note, is the difference in route of administration of the antagonist. Here, L-152-804 was injected centrally (ICV), whereas the novel Y5 antagonist, Lu AA33810 was injected systemically (IP). In addition, previous studies using L-152-804 have employed systemic administration of the antagonist (Woldbye et al. 1998; Kanatani et al., 2000; Schroeder et al., 2005). Second, reinstatement tests in experiment 2A (L-152-804) included an "off" period in which animals remained in

operant chambers for two days under food-deprived or *ad libitum* conditions without any activation of the operant system, making a direct comparison between the two studies somewhat problematic. Finally, experiment 2A (L-152-804) consisted of a 48 hr food-deprivation period versus 21 hr (experiment 2B; Lu AA33810). It is possible that there are neuroadaptations that occur over 48 hr that might affect the involvement of NPY-dependent mechanisms in the reinstatement effect. In conclusion, it appears that L-152-804 was ineffective in the current study perhaps due to methodological issues. In contrast, the potent, selective and novel Y5 receptor antagonist Lu AA33810, was highly effective in the attenuation of FD-induced reinstatement of heroin-seeking behavior. These findings indicate that the Y5 receptors play a critical function in processing the conditioned properties of reward following acute FD stress.

One possible role for Y5- receptor antagonism is that it reduces the "hunger"-like state driven by food-deprivation. Activation of "hunger" pathways traditionally found in the hypothalamus, could modulate the saliency of reward-associated cues for food or drug. Y5 receptor antagonism might have dampened hunger signals initiated by FD and thus inhibit FD-induced reinstatement of drug-seeking. Indeed, treatment with the non-specific Y5-receptor antagonist, CGP71683A, significantly suppressed spontaneous nocturnal feeding and fasting-induced feeding (Kask, Vasar, Heidmets, Alliments, & Wikberg, 2001). However, the role of Y5 receptor in the control of feeding has recently been questioned. Accumulating reports indicate that NPY Y5-receptor by itself does not have a major role in feeding behavior. For example, the Y5-receptor antagonist, NPY5RA-972, potently suppressed food intake induced by the Y5-receptor agonist cPP, but failed to attenuate NPY-induced feeding or

spontaneous food intake (Turnbull et al., 2002). Similarly, the Y5-receptor antagonist used here, Lu AA33810, was shown to have no effect on NPY-evoked food consumption (Walker et al., 2009). In addition, acute administration of a spironolactone Y5-receptor antagonist did not significantly reduce food intake or body weight in high-fat diet-fed mice, while treatment with a Y1-receptor antagonist resulted in a robust suppression of food intake and body weight (Mashiko et al., 2009). Finally, NPY Y1 and Y5-receptors are found throughout the hypothalamus, as expected (Wolak et al., 2003). However, food deprivation seems to increase the expression of Y1 receptor mRNA, while the expression of Y5 receptors is decreased (Xu, Kalra, Moldawer, & Kalra, 1998; Widdowson, 1997). It therefore seems that Y1 and Y5 receptor co-activation is required for energy homeostasis at the level of the hypothalamus (Mashiko et al., 2009).

In conclusion, although we cannot completely rule out a hunger-suppression mechanism underlying the effects of the Y5 receptor antagonist on FD-induced reinstatement of heroin seeking, the findings described above make this interpretation highly unlikely.

Another way for the Y5 receptor to modulate the reinstatement effect is through the mediation of anxiolytic responses. The present results, together with findings from previous studies, suggest that Y5-receptor antagonism could be involved in *reducing* anxiety-producing states, an effect opposite to the one suggested for Y1 receptor antagonism described above. Systemic injections of Lu AA33810 at doses shown to decrease Y5 receptor agonist-induced feeding have been shown to produce anxiolytic and anti-depressant-like effects in rats (Walker et al., 2009),

findings which are opposite to our results with Y1-receptor antagonism (experiment 1). These findings suggest that antagonism of the Y5-receptor could counteract the stressful effects of FD. However, identifying the underlying mechanism for this effect appears problematic. The amygdala has been identified as the critical area for the anxiolytic effects of NPY (Heilig, 2004). Moreover, the amygdala contains Y1 and Y5 receptors, however, Y5 receptor expression within the amygdala appears to be primarily restricted to the basolateral amygdala (BLA; Wolak et al., 2003). It has been shown that activation of Y5 receptors produces effects similar to those found with Y1 receptor activation, where Y5-receptor agonists show an anxiolytic effect similar to that of ICV administration of NPY (Sorensen et al., 2004). However, interestingly, it has been reported that the anxiolytic effects of NPY are specific to the BLA through *activation* of Y5, but not Y1 receptors (Sajdyk, Schober, & Gehlert, 2002), thus ruling out the BLA as the critical target area for Lu AA33810 in our study.

Finally, Lu AA33810 might modulate the putative role of NPY transmission in the hedonic properties of reward as opposed to a homeostatic regulation per se. As previously mentioned, Y5 receptor antagonists have been shown to be involved in drug-reward-related behavior (Schroeder et al., 2005; Sorenson et al., 2008), and thus it is possible that Y5 receptors modulate the effect of FD on drug-seeking through the reward pathways, and more specifically, the mesocorticolimbic dopaminergic (DA) pathway. DA projections from the ventral tegmental area (VTA), to nucleus accumbens (NAc) and to the prefrontal cortex (PFC) constitute the mesocorticolimbic pathway, which is one of the pathways thought to orchestrate motivational, affective

and learning-based reward processing (Kelley & Berridge, 2002; Berridge & Robinson, 2003). It is well-documented that DA projections in the mesocorticolimbic system play a role in mediating the rewarding properties of natural rewards such as food and sexual stimuli and of drugs of abuse (Kelley & Berridge, 2002). Periods of food restriction and deprivation are thought to modulate drug-reward through changes in dopaminergic signal transmission. For example, food-restricted rats have greater NAc DA release when injected with systemic cocaine (Rouge-Pont, Marinelli, Le Moal, Simon, & Piazza, 1995), as compared to rats that had *ad libitum* access to food. In addition, chronic food restriction (40-50% of *ad libitum* food intake) increased sensitivity to lateral hypothalamic ICSS when pretreated with addictive substances like amphetamine (Cabeza de Vaca & Carr, 1998), as demonstrated by a lowering of ICSS threshold in food-restricted animals. Finally, it appears that acute food-deprivation (24-36 hrs) modulates striatal dopamine reuptake transporter (DAT) function, as indicated by a decrease in V_{max} of DA uptake in fasted rats (Patterson, Brot, Zavosh, Schenk & Figlewicz, 1998). It has been suggested that the effects of food restriction or acute deprivation on reward processing in the mesolimbic DA system may depend on endocrine signals and/or neuropeptidergic signals (NPY, leptin, ghrelin) that change in response to ingestive manipulations (Carr, 2002). This idea is supported by studies that have shown that NPY stimulates DA release in the nucleus accumbens (Ault, Radeff & Werling, 1998, Sorenson et al., 2009). Interestingly, it appears that the role of NPY in mediating homeostatic mechanism is distinct from its hedonic properties, as injections of NPY itself elicits reward-related behavior (e.g. CPP), but not feeding, when injected in the NAc, and both behaviors

when injected in the hypothalamus (Brown, Coscina & Fletcher, 2000). This suggests that NPY transmission might have reward-related properties itself. Thus, during food deprivation-induced reinstatement of drug seeking, activation of specific receptor subtypes, namely Y5, could alter the saliency and efficacy of cues associated with drugs of abuse. Consistent with this suggestion, it was reported that NPY Y5-receptor protein and mRNA are strongly expressed in mesocorticolimbic regions such as the NAc (Wolak et al., 2003) and VTA (Parker et al, 1999; Morin & Gehlert, 2006). However, a specific role for Y5 in conjunction with DA receptor localization or cell functionality has not been explicitly identified.

Findings from experiment 1 and 2 demonstrated that NPY transmission might be critical in mediating the effects of FD on reinstatement of drug-seeking via a specific NPY receptor activation, namely, the Y5 receptor. These findings lead to the question of whether the activation, or suppression, of peripheral signaling (i.e. ghrelin, leptin) during food deprivation, resulting in increased NPY transmission, is necessary for the expression of reward-related behavior. In order to investigate this issue, we assessed whether ghrelin itself is involved in motivational aspects of drug-taking behavior, and whether it is critically involved in food-deprivation induced reinstatement of drug-seeking behavior.

The role of ghrelin in heroin taking and seeking behavior

Ghrelin, the endogenous GHS-R agonist, activates GHS-R receptors and mRNA throughout hypothalamic sites such as the ARC, PVN, brainstem and also in other regions such as the amygdala and VTA (Zigman, Jones, Lee, Saper & Elmquist,

2006). It is thought, that in addition to its role in metabolic states, ghrelin can enhance the incentive value of reward-related stimuli. This was recently demonstrated by the increased activity observed in areas associated with reward processing and appetitive behavior in humans that had an overnight fast (12 hr) prior to ghrelin injections (Malik, McGlone, Bedrossian & Dagher, 2008). Experiment 3 evaluated the ability of ghrelin, a hormone that is secreted during food restriction, to increase motivational aspects of drug reward. The PR schedule of reinforcement used was intended to measure animals' motivation to obtain the drug reward. Results demonstrated that ICV infusions of ghrelin at doses shown to increase food intake, enhanced the motivation to self-administer heroin, as measured by the breakpoint attained as well as the number of active presses performed (a measure of drug seeking behavior; experiment 3A). Moreover, ghrelin appeared to elicit reinstatement of drug-seeking behavior as demonstrated by higher breakpoint achieved under extinction conditions in FD animals under a PR schedule of (conditioned) reinforcement (experiment 3B). Although statistical significance was not achieved for experiment 3B, it is important to note that there was a clear trend for ghrelin-induced reinstatement. This effect is impressive, as reinstatement on PR schedule is difficult to demonstrate since a relatively small change in breakpoint represents a large difference in active lever presses. This effect, although not statistically significant, was observed under extinction conditions with only the presence of drug-associated cues to support the behavior. Reasons for lack of statistical significance may include high variability and low sample size in each treatment group, thus we believe that the present results merit some consideration.

Findings from experiment 3 are important as they demonstrate that ghrelin has the ability to increase the rewarding value of addictive drugs, but can also enhance the motivational and the conditioned properties of drug-associated cues. These effects might be due to a "hunger"-like state triggered by ghrelin transmission. As we have elaborated above, such states, usually driven by food restriction or food deprivation, would result in increased drug taking and seeking behaviors. However, it is also possible that these effects are due to mechanisms that are not associated with energy homeostasis, as we have previously suggested for the NPY-related findings.

Ghrelin acts directly on many extra-hypothalamic brain targets and thus can modulate the reinforcing effects of food, and drugs of abuse as presented here. It has been shown that ghrelin increases the hedonic properties of palatable food (Perello et al., 2009), and alcohol reward (Jerlhag et al., 2009). GHS-R1a are expressed in DA-containing neurons in the VTA and the latero-dorsal tegmental nucleus (LDTg; Abizaid et al., 2006b; Zigman et al., 2006), important structures for the rewarding and reinforcing effect of drugs of abuse. Initial studies demonstrated that intra-VTA and, interestingly, intra-NAc injections of ghrelin increased intake of standard chow in rats (Naleid, Grace, Cummings & Levine, 2005). Moreover, systemic administration of ghrelin in rats was shown to increase DA levels in the shell of the NAc, a subdivision that is associated with hedonics and reward, and was shown to increase locomotor activity and extracellular levels of accumbal DA in mice when administered ICV (Quarta et al., 2009; Jerlhag et al., 2006). Interestingly, ghrelin administration into either the VTA or LTDg, in mice, has been shown to acutely increase locomotor activity and extracellular concentration of accumbal DA (Jerlhag et al., 2007). Thus,

release of DA in the NAc, possibly in the shell region, via activation of DA neurons in the VTA by ghrelin, may constitute the underlying mechanism through which ghrelin exerts its motivational effect. This suggests that ghrelin might participate in the initiation of motivated behaviors by shifting incentive value of reward, an effect perhaps dependent upon DA-ergic transmission (Jerlhag et al., 2006; 2007).

Based on the demonstrated ghrelin-induced increases in drug-taking found here, it was hypothesized that antagonism of ghrelin receptors would inhibit drug-self-administration. Moreover, since an effect on reinstatement of extinguished drug seeking by exogenous ghrelin administration was indicated, it was hypothesized that antagonism of its receptors would inhibit FD-induced reinstatement. Results from experiment 4 demonstrated that this was not the case: antagonism of ghrelin receptors had no effect on on-going heroin self-administration (experiment 4A). In addition, results demonstrated that treatment with the ghrelin receptor antagonist was ineffective in modulating FD-induced reinstatement (experiment 4B). It is important to note that an increase in active lever presses (not statistically significant) in ghrelin antagonist treated animals in the *ad libitum* condition was observed. We have noted that some animals infused with the antagonist during self-administration and reinstatement experiment demonstrated a strong seizure response post-injection, which subsided 10 minutes later, which might have triggered the response on the previously drug associated lever. This is thought to represent a side effect of the antagonist that has yet to be reported from other studies.

In conclusion, ghrelin appears to modulate drug-taking behavior. This effect might involve interactions with the mesolimbic DA system, where increasing the

incentive value of drug reward, as shown with food reward. However, it seems that activation of ghrelin receptors is sufficient to induce increases in drug taking and seeking behavior, but is not necessary, as treatment with ghrelin receptor antagonist had no effect on drug taking or FD-induced reinstatement of extinguished heroin seeking.

Caveats

Some limitations of the present study should be considered when assessing the results reported here. First, and most importantly, the interpretations of possible underlying mechanisms for the results of the present study must be considered with caution due to the lack of site-specific injections. Second, ghrelin-induced reinstatement of heroin seeking could reflect a conditioned association between heroin and ghrelin, since animals were pre-exposed to ghrelin during the self-administration phase (Experiment 3). This, however, does not seem to be the case, since rats that were not exposed to ghrelin during the self-administration phase demonstrated similar behavior during reinstatement tests. Third, the seizure side-effects observed with the ghrelin receptor antagonist (Experiment 4), might suggest that the compound itself could have targeted hippocampal regions and resulted in cell damage, as ghrelin was shown to protect against neuronal damage caused by seizures (Xu et al., 2009). Finally, limited sample size and high variability found in Experiment 1 using the Y1-receptor antagonist could suggest that a larger number of subjects would have resulted in a robust Y1-receptor antagonist-enhanced reinstatement within the FD conditions.

Future Directions

Since the distribution of NPY and ghrelin receptors is widespread, future experiments should investigate localization of the observed effects in relevant brain areas. For example, either intra-amygdala (for Y1-receptor inhibition), or intra-NAc and intra-VTA (for Y5 and ghrelin-receptor inhibition, respectively) injections, during FD-induced reinstatement of drug seeking would be needed in order to consolidate our interpretations. In addition to localization, in order to evaluate the role of orexigenic hormones in drug-taking and drug-seeking, it would be of interest to directly measure changes in extracellular DA levels during on-going self-administration and the FD-induced reinstatement tests following orexigenic peptide signal blockade. Finally, it would be interesting to compare the effects of orexigenic hormones during periods of chronic food-restriction and acute food-deprivation, as they have been shown to induce different circulating hormone levels and peptide mRNA expression in the hypothalamus (Johansson et al., 2008; Bi et al., 2003). This differentiation is important, as the effects of chronic food-restriction might cause neuroadaptations that are different from those produced by acute FD, which could impact motivational and reinforcing features of goal-directed behavior. This notion is supported by findings that have shown that unlike chronic FR, acute FD (48 hr) is ineffective in increasing sensitivity to ICSS in the LH (Fulton et al., 2000). Furthermore, chronic FR was shown to result in a 32% reduction in V_{max} of DA reuptake in the striatum (Zhen, Reith & Carr, 2006), as compared to a 13% reduction following 36 hr of acute FD (Patterson et al., 1998). Thus, it is postulated that although both chronic FD and acute FD alter reward-related behavior and DA-

associated reward processing, it is possible that the mechanisms that underlie each dietary manipulation are distinct. Moreover, the ecological nature of these feeding regiments is important to consider when trying to link them to vulnerability to drug addiction in humans, as chronic FR is used as a model of dieting-induced changes in sensitivity to drug-taking, whereas acute FD is a model of stress-induced relapse to drug-seeking.

Conclusion

In the present thesis we tested the hypothesis that orexigenic hormones known to be involved in feeding would modulate reward-related behaviors in a direction similar to that evidenced for feeding behavior. Results demonstrated that this direct relationship was not absolute, as orexigenic hormones have a complex neurobiological network, which controls various behaviors concomitantly, all aimed at increasing the organism's survival probability. Experiment 3 and 4 demonstrated that ghrelin receptor activation is sufficient, but not necessary to modulate the reinforcing, motivational, and conditioned properties of drugs of abuse. These findings are somewhat surprising since results from experiment 2 indicated that NPY Y5-receptors are essential for FD-induced reinstatement, and thus it was expected that ghrelin signaling during reinstatement would play a major role, as it is one of the main modulators (in addition to leptin) of NPY expression in the hypothalamus. Accordingly, it is postulated that the modulation by ghrelin and NPY of drug-related behaviors might occur outside a traditional homeostatic regulatory system in the hypothalamus. Results from the present thesis may have implications for the elucidation of the putative common neuronal circuitry underlying eating disorders and

substance abuse, which might reflect a dysregulation in general reward functioning, as opposed to a "simple" homeostatic disturbance.

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APPENDICES

APPENDIX A: Food-intake tests

Table 1.

Experiment 1. Mean (+/-*SE*) food intake (g) following injections of NPY Y1-receptor antagonist, BIBO 3304.

	Baseline	0.0 nmol/rat/ICV	10.0 nmol/rat/ICV
1 hr	4.33 (0.42)	4.25 (0.42)	3.15 (0.58)

3 hr	10.90 (0.92)	10.10 (0.77)	8.85 (0.89)
24 hr	40.77 (1.73)	39.30 (2.75)	32.65 (2.79)

Table 2.

Experiment 2A. Mean (+/-*SE*) food intake (g) following NPY Y5-receptor antagonist, L-152-804.

	Baseline	0.0 µg/rat/ICV	20.0 µg/rat/ICV
1 hr	3.08 (0.98)	3.17 (0.83)	2.17 (0.93)
3 hr	6.50 (2.43)	3.83 (0.17)	4.50 (2.02)
24 hr	34.33 (5.28)	26.83 (10.17)	29.16 (5.18)

Table 3.

Experiment 2B. Mean (+/-*SE*) food intake (g) following NPY Y5-receptor antagonist, Lu AA33810 inhibition of Y5-agonist induced feeding.

	Vehicle Baseline	0.0 mg/kg/IP	Antagonist Baseline	30.0 mg/kg/IP
1 hr	4.16 (1.04)	10.67 (2.33)	5.87 (1.23)	5.75 (1.03)
3 hr	11.83 (1.74)	19.33 (1.67)	12.75 (2.56)	14.75 (2.14)

24 hr	36.5 (1.07)	33.33 (1.76)	40.5 (5.73)	46.25 (2.95)

Table 4.

Experiment 3. Mean (+/-*SE*) food intake (g) following injections of ghrelin.

	Baseline	0.0 µg/rat/ICV	1.5µg/rat/ICV	3.0 µg/rat/ICV
1 hr	4.4 (0.29)	4.6 (0.42)	4.8 (0.49)	6.0 (0.87)
3 hr	9.9 (0.56)	9.0 (1.3)	8.7 (0.72)	11.8 (2.31)
24 hr	34.0 (0.29)	31.0 (0.29)	27.5 (0.29)	34.7 (0.29)

Table 5.

Experiment 4. Mean (+/-*SE*) food intake (g) following injections of ghrelin receptor antagonist.

	Baseline	0.0 µg/rat/ICV	20.0 µg/rat/ICV
1 hr	2.34 (0.81)	2.3 (0.25)	0.0 (0.0)
3 hr	7.85 (1.81)	5.5 (0.50)	2.3 (0.75)
24 hr	28.43 (4.01)	25.8 (6.75)	32.5 (2.50)

APPENDIX B: Experiment 1B. The effects of Y1 receptor antagonism on performance in the elevated plus maze in food deprived rats.

Mean (+/-*SE*) percentage of time spent in the open arms and number of open arm approaches (in 5 min) on the elevated plus maze following injections of Y1-receptor antagonist, BIBO 3304.

	0.0 nmol/rat/ICV	10.0 nmol/rat/ICV
Percentage of time spent in open arm (TO)	44.4 (9.64)	18.6 (4.40)
Number of Open Approaches (OA)	7.0 (0.55)	9.6 (1.21)