OREXIN AND NPY INTERACTIONS IN FEEDING BEHAVIOR

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Dedication

I dedicate this to my parents, Cristela Torres and Gilberto Ferri. Their love and support will never be forgotten. Their desire, determination and dedication were a standard upon which the achievements of my goals were achieved. Their wish for me to obtain higher education has been accomplished.

Bendición!

Orexin and NPY Interactions in Feeding Behavior

By

Erwin David Ferri

Obesity results from an imbalance between energy intake and energy expenditure. The central nervous system (CNS) has a complex interconnected circuitry that regulates feeding behavior. The hypothalamus is the principal region in the central nervous system regulating energy intake and energy expenditure. Important advances have been made identifying hypothalamic neuronal networks, neuropeptide transmitters and the discovery of circulating peptides that send signals to the brain regarding the body's nutritional status. However, the full set of neuronal pathways that initiate changes in ingestive behavior or energy expenditure remain undefined.

This dissertation examines the roles of two specific neuropeptides, orexin A (OXA) and neuropeptide Y (NPY) in food regulation. Several lines of evidence suggest that orexin and NPY interact in modulating feeding behavior and these dissertation studies further substantiate this premise. Previous research conducted in this laboratory showed that subthreshold doses of OXA and NPY agonists stimulate feeding when administered simultaneously. The current set of studies shows effects of individual and simultaneous central administration of subthreshold doses of NPY and OXA antagonists on feeding.

The first study established dose-response parameters for both OXA and NPY antagonists. The OXA antagonist was injected into the lateral hypothalamic area (LHa) whereas the NPY antagonist was injected into the hypothalamic paraventricular nucleus (PVN). The objective was to ascertain sub-threshold doses for both of these neuropeptides antagonists within these brain sites. The study showed that the subthreshold dose for both the NPY and the orexin antagonist was 100 pmol.

The second study was the central administration of both subthreshold doses of the NPY antagonist and the orexin antagonist simultaneously, within the PVN and LHA respectively. Individual administration of subthreshold doses of the orexin and NPY antagonists in the LHA and PVN respectively caused no inhibition of food intake, whereas simultaneous administration of subthreshold doses of the orexin and NPY antagonists significantly inhibited food intake. Significant differences ($p < 0.001$) were obtained at all time points (0-1 h, 0-2 h, 0-4 h and 0-24 h) comparatively with vehicle.

The experiments conducted further substantiate the premise of a functional relationship between OXA and NPY that involves corresponding pathways between the LHA and the PVN.

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Introduction

Obesity is one of the major public health problems in industrialized and developing countries. Obesity is often associated with chronic diseases such as hypertension, hyperlipidemia, insulin resistance and type II diabetes (1). The consequences of obesity are also associated with increased risk of coronary heart diseases and a higher incidence of several major cancers (breast and colon) than normal weight subjects (1). Obesity is the result of a discrepancy between energy intake and energy expenditure (2). Treatment of obesity has proven difficult due to the fact that the regulation of energy intake and expenditure is controlled through multiple and complex ways that are not fully understood.

The complex neuronal circuitry in the CNS concerning food regulation has been a primary research foci regarding obesity. Although there have been many scientific breakthroughs that have created a better understanding of these issues, there are many areas that have as of yet to be explained.

One of the most highly researched regions of the CNS regarding obesity is the hypothalamus. This in due to the many pathways converging on the hypothalamus and the neuropeptides and neurotransmitters expressed in this small brain region that influence feeding. It integrates neural signals via vagal stimuli, chemical and hormonal signals from the gut and adipose tissue and sensory signals from higher centers in the brain. The hypothalamus anatomical position facilitates its connections with afferent input from the periphery, via the brainstem, and higher brain centers that are involved in the control of food intake and energy homeostasis.

Central Food Regulation

Research concerning the CNS and the physiology of energy homeostasis regarding obesity is ongoing. The hypothalamus is the principal region in the central nervous system that regulates energy intake and energy expenditure. Important advances have been made identifying hypothalamic neuronal networks, neuropeptide transmitters and the discovery of circulating peptides that send signals to the brain regarding the body's nutritional status. However, the neuronal pathways that actually initiate changes in ingestive behavior or energy expenditure are still largely unknown.

The central nervous system (CNS) has a complex interconnected circuitry which regulates feeding behavior. Early theories concerning CNS food regulation emerged after discrete lesions of the brain permitted the evaluation of brain function. Bilateral electrolytic lesions of the ventromedial hypothalamus (VMH) caused marked hyperphagia and obesity in rats (3) suggesting a reduction in sensitivity to incoming signals of satiety. In contrast, bilateral lesions in the lateral hypothalamus (LHA) were found to cause aphagia (absence of eating) (3, 4). Animals with LHA lesions no longer detected hunger signals leading to starvation. Together these studies established the dual center hypothesis in which a satiety center in the VMH was thought to suppress activity in a hunger center located in the LHA (3, 4). Subsequent observations have reinterpreted these findings and it is now known that food intake is not controlled solely by the hypothalamic hunger and satiety centers.

Neuropeptide Y (NPY)

Neuropeptide Y is a 36-amino acid neuropeptide member of the pancreatic polypeptide family that includes peptides YY and PP (5, 6) and is one of the most abundant and widely distributed peptides within the CNS. The effect of NPY on feeding behavior and regulation is of utmost interest. The most noticeable effect of NPY is the stimulation of feeding after central administration (6). Central administration of NPY into PVN produces a powerful and prolonged increase in food intake (7) and when administered chronically, NPY produces hyperphagia, decreased thermogenesis and obesity (8).

Neuropeptide Y exerts its effects in a wide range of both peripheral and central targets. Some of the peripheral targets include blood vessels, the heart, the gastrointestinal tract, the kidney, pancreas, thyroid glands and sympathetic, parasympathetic and sensory nerves (9). Central NPY influences pituitary hormone release, behavior and central autonomic control (8, 9). Neuropeptide Y is found throughout the cerebral cortex and forebrain nuclei, but is contained in a variety of neurons in the hypothalamus, brainstem and spinal cord (9). NPY is implicated in several physiological functions including the regulation of feeding (10), the control of learning and memory (11), locomotion (12), body temperature regulation (13), sexual behavior (14), emotional behavior (15), neuronal excitability (16), cardiovascular functions (17), circadian rhythms (18) and hormone secretion (19).

Current theories suggest that peripheral signals reflecting long-term energy balance are processed centrally, and lead to modulation of day-to-day energy intake and expenditure. Specific regions of the brainstem and hypothalamus nuclei are important in coordinating these peripheral satiety and adiposity signals (20, 21).

As part of a more specific network, the hypothalamus regulates short and long-term food intake via various orexigenic and anorectic neurotransmitters (21). In the arcuate nucleus (ARC), LHA and PVN, the roles of two specific feeding regulation neuropeptides, OXA and NPY, have been characterized in rodent models (22, 23). Studies have shown that both orexin (produced in the LHA) and NPY (produced in the ARC) affect food intake and are essential in energy homeostasis (24, 25). The ARC, which is located next to the third ventricle and the median eminence, is an important area of interaction between peripheral signals and the brain (26).

Neuronal projections from the ARC to other areas of the brain, including the PVN, ventromedial hypothalamic nucleus (VMN), dorsomedial hypothalamic nucleus, LHA, and perifornical area are thought to mediate the effects of the ARC neuronal system on energy homeostasis (27). Neuropeptides produced in these regions are released to stimulate or inhibit feeding behavior (26, 27). Other regions of the brain, such as the nucleus of the solitary tract, the nucleus accumbens, and the ventral tegmental area, have also been suggested to be important for mediating food reward (28).

The major sites of NPY expression in the hypothalamus are in the ARC (29, 30). The ARC in the hypothalamus contains two subsets of neurons that control food intake. The first group contains neurons expressing the orexigenic peptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP). The second group expresses anorexigenic peptides derived from pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) (28). POMC is a precursor to the anorectic α -melanocyte stimulating hormone (α -MSH). Activation of these neurons has the effect of decreasing food intake and increasing energy expenditure (30). The arcuate POMC and NPY/AgRP neurons constitute a functional unit in which neural inputs to NPY/AgRP cells may rapidly affect both NPY/AgRP and POMC neurons (30).

Arcuate NPY gene expression is modulated in response to alterations in energy balance (31). NPY immunoreactivity is highest in the PVN, almost exclusively in nerve terminals, a large portion of which arise from cell bodies in the ARC (31). This ARC–PVN NPYergic pathway has been shown to be highly sensitive to energy status. ARC NPY mRNA and PVN NPY levels increase in response to food deprivation (32) or food restriction (33-35) and normalize after re-feeding (36). Both acute food deprivation and chronic food restriction reduced body weight and circulating leptin levels and resulted in increased ARC NPY and decreased ARC POMC gene expression. These NPY neurons co-express leptin receptor mRNA (37, 38), and NPY gene expression is downregulated by leptin administration (38).

To date, six NPY receptor subtypes have been characterized, termed Y1 through Y6 (39) and have been identified as members of the G protein-coupled receptor family. Pharmacological studies using NPY analogs have suggested that it is likely that NPY-Y1 and or NPY-Y5 receptors are involved in the control of feeding (39). The NPY Y1 and Y5 receptors are found in hypothalamic areas corresponding to those in which NPY administration evokes a feeding response in rats.

The NPY receptor mediating feeding has been described as 'Y1-like', since agonists selective for this receptor stimulate feeding when given centrally (40,41). Many physiological actions of NPY are likely mediated by Y1 receptors as suggested on the basis of knockout (KO) studies (42, 43), and the use of selective agonists and antagonists (44).

Studies using Y1 receptor antisense oligodeoxynucleotides have demonstrated that although feeding was not decreased in ICV injected antisense-treated rats, hypothalamic injection of Y1 receptor antisense could suppress NPY- induced food intake (45). These data suggests that the Y1 receptor is a crucial mediator of the feeding behavior caused by NPY (46).

Another indication that Y1 receptor signaling plays a prominent role in the stimulation of feeding and obesity comes from the observation that changes in feeding behavior and energy balance induce a marked plasticity in the Y1 receptor function and expression in specific regions of the hypothalamus (45,47).

Compensatory changes in Y1 gene expression happen when NPY synthesis and release are increased or reduced by neuronal activity or peripheral hormones (48). Plasticity of Y1 gene expression may be induced by positive or negative states of energy balance, reproductive activity and brain injuries, including prolonged stressful stimuli and epileptic seizures (45).

Recent findings point toward the Y5 receptor as playing a major role in food regulation (49, 50). Y5 receptor mRNA is widely distributed in CNS but less abundantly than the Y1 mRNA (51). In the rat brain, distribution of Y5 receptor mRNA was primarily restricted to specific hippocampal, hypothalamic, and associated regions of the rat forebrain (51). The Y5 receptor is expressed at relatively high levels in the LHA and its distribution in the brain indicate that it is localized in areas consistent with a role for of this receptor in NPY-induced feeding (52-54). However, knockout (KO) of the Y5 receptor raises doubts concerning this assumption. (49).

Studies using Y5 receptor knockout (KO) mice demonstrate that this receptor mediates at least some of the effects of centrally administered NPY (49, 54). Central administration of Y5 antisense oligodeoxynucleotides resulted in weight loss and a decrease in food intake, and it inhibited the increase in food intake after ICV injection of NPY in rats (56, 57). Inactivation of the Y5 gene demonstrated that the Y5R is not required during appropriate feeding or during food deprivation.

Younger Y5 KO mice eat and grow normally but gradually develop late-onset obesity characterized by increased body weight, food intake and adiposity (49). Food intake in mice with ICV administration of NPY was either reduced or absent and fasting induced re-feeding response was unchanged (49). Absence of NPY lessened the obesity syndrome of leptin deficient (*ob/ob*) mice but these effects were not mediated by NPY signaling through the Y5 receptor (49), possibly because Y5 null *ob/ob* rats are equally obese. These data suggest that although Y5 contributes to feeding induced by centrally administered NPY, it is not a critical physiological feeding receptor in rats (49).

Which of these two receptors is more important in the regulation of feeding is currently unclear. Studies have provided evidence that both Y1 and Y5 selective antagonists inhibit NPY-induced or physiological food intake (58, 59). However, the lack of specific Y5 antagonists and the limited number of Y1 receptor antagonists has hampered the determination of which particular NPY receptor should be postulated as the primary mediator of feeding behavior (49).

Studies with existing Y1 antagonists suggest a pivotal role for this receptor in feeding regulation (60, 61). The first Y1 receptor antagonist that became available was BIBP3226 (62), which inhibits NPY induced feeding in rats. The antagonist BIBO 3304, 1229U91 (also known as GR231118), J-104870 and LY357897 also act on this receptor subtype and cause similar reductions on NPY-induced intake. (63-67).

There has been a gradual development of selective Y5 receptor antagonists (68, 69). Selective Y5 antagonists, such as CGP 71683A (70), GW438014A (71), and FMS586 (72) have been shown to inhibit spontaneous intake, fasted food intake and NPY-induced food intake in rats.

Reduced body fat stores in adiposity tissue induce adaptive changes in neural systems that regulate both food-seeking behavior and satiety perception. The 'adiposity negative-feedback' model of energy homeostasis is based on the premise that information is transmitted to the brain regarding changes in body fat mass and that the brain responds with adaptive adjustments of energy balance to stabilize fat stores (73-76). In force-fed animals, the increased daily intake of calories causes weight gain (77). When food is given ad libitum, these previously force-fed animals tend to eat smaller meals than normal or no meals at all until normal adiposity is restored. Conversely, response to weight loss increases motivation to find food and the size of individual meals tend to increase until energy stores are replenished (76, 77). Compensatory responses to weight fluctuations caused by acute changes in food intake or energy expenditure are the basis of long-term stability of adiposity (77).

Insulin and leptin fit the criteria for negative-feedback signals. They both circulate in balanced proportion to body fat content and enter the brain. By acting on neuronal systems implicated in energy homeostasis they promote weight loss. Inhibition of leptin and insulin increases food intake and body weight (78). Both hormones promote negative energy balance through their effects in the brain.

Leptin is a hormone secreted by adipose tissue that acts to regulate long-term appetite and energy expenditure by signaling the state of body fat reserves (79, 80). Leptin acts centrally to inhibit NPY synthesis leading to the reduction of food consumption and food intake (81, 82).

Leptin administration has also been shown to suppress NPY gene expression in the ARC and NPY release from hypothalamic fragments in vitro (82) thereby suggesting that suppression of feeding by leptin may, in part, be mediated by hypothalamic NPY.

Animals that lack the gene for synthesizing leptin (*ob/ob* mice) are hyperphagic and obese (79, 80). Administration of leptin in obese *ob/ob* mice effectively reduces hyperphagia and obesity (83). In contrast, obese mice lacking leptin receptors *(db/db*) do not respond to central administration of leptin (83, 84).

The expression of ObRb (long form of leptin receptor) is highest in regions of the hypothalamus that mediate food intake and energy homeostasis (85, 86). In the ARC, leptin exerts some of its effects by acting on at least two distinct populations of neurons. NPY and AgRP mRNA expression is decreased due to the effects of leptin (85, 86). NPY-containing neurons have ObRb receptors and leptin acts on these neurons to inhibit expression of NPY and its release. By contrast, leptin increases the expression of POMC mRNA (87).

The pancreatic hormone insulin enters the brain through blood circulation and acts in the brain to reduce energy intake. Insulin is essential for stimulating glucose uptake and metabolism in peripheral tissues (88). Central administration of insulin decreases food intake and body weight, and insulin receptors are concentrated in brain regions that are involved in controlling the intake of food (89).

Weight gain affects insulin sensitivity and insulin varies according to body fat stores (90). As weight increases, insulin secretion must increase in both the basal state and in response to meals to compensate for insulin resistance if normal glucose homeostasis is to be maintained (91, 92). Failure to achieve this adaptive increase of insulin secretion causes hyperglycaemia, and probably contributes to the association of type 2 diabetes with obesity.

Food intake regulation requires the integration of various neural and hormonal signals from a peripheral satiety system, which includes the gut and adipose tissue (93). Both distension of the stomach as well as caloric content of nutrients are involved in the regulation of food intake (94). Meal size appears to be regulated by afferent signals that originate in the oral cavity and the gastrointestinal (GI) tract and are transmitted mainly by the vagus nerve to the nucleus of the solitary tract (NTS) in the brainstem. The NTS relays this information and communicates with higher hypothalamic centers involved in the regulation of food intake and energy expenditure (95, 96, 97).

Short-term signals from the gut primarily regulate satiety while long-term signals from adipose tissue encode energy stores (98, 99, 100). The GI produces and secretes biologically active polypeptides/hormones that exert their actions via circulation or by acting directly in the CNS (101-103). Two of these, ghrelin and CCK, exert contrasting effects on food intake regulation.

Ghrelin, produced in the stomach, is the only gut hormone known to stimulate hunger and increase food intake [104, 105, 106]. Once released, ghrelin enters circulation via the bloodstream, crosses the semi intact blood brain barrier and penetrates the ARC within the hypothalamus. Small amounts of ghrelin may be produced within the hypothalamus itself and this could directly activate NPY/AgRP neurons and neurons in the LHA (107).

Chronic administration of ghrelin produces hyperphagia and generates weight gain in rodents (108). Intra-cerebroventricular (ICV) administration of ghrelin in rodents increases short-term food intake. Neuropeptide Y and AgRP gene expression in the ARC are augmented following ICV administration of ghrelin indicative of the effects of ghrelin on appetite (109). ICV injection also induces *cfos* expression in the lateral hypothalamus, specifically in orexin-containing neurons suggesting a central pathway linking ghrelin and the orexins (110, 111). Electrophysiological studies showed that in addition to the activation of NPY neurons in the ARC, pro-opiomelanocortin (POMC) neurons are also inhibited (112). Ghrelin, therefore, acts on the hypothalamus by direct activation of neurons containing orexigenic peptides, NPY and orexin, and by inactivation of POMC neurons (113).

Cholecystokinin (CCK) is a satiety signal that provides information about the chemical (smell, taste and texture) properties of food. It is released after food intake (meals) in response to fat and protein ingestion (114-116). Cholecystokinin acts to facilitate nutrient absorption from the gut and may also act locally to augment the mechanical signals of gastric and intestinal distension to the hypothalamus (117,118).

The satiety signal CCK binds to CCK receptors on the vagus nerve, which projects to the NTS in the brainstem (119-121). The NTS relays this information to the hypothalamus where the signals are projected to the cortex and gastric distension is perceived. When administered either centrally or peripherally CCK is known to suppress feeding (120). ICV administration of CKK has been shown to be highly effective in decreasing food intake (121). Long-term administration of CCK demonstrated that its food inhibitory effect is short-term. According to these studies CCK continued to cause inhibition of food intake but it also induced an increase in meal frequency therefore not causing a reduction in weight (122).

Orexin

Orexin A (OXA, hypocretin-1) and Orexin B (OXB, hypocretin-2) are neuropeptides that were isolated from the rat hypothalamus by two independent groups of investigators in 1998 (123, 124). The first group of investigators named these peptides hypocretins because of their hypothalamic localization and their structural similarity to secretin. The second group used the term orexin because of their abundant expression in the hypothalamic feeding centers and their apparent involvement in the control of food intake.

Orexin A, a 33 amino acid peptide with two disulfide bonds, and OXB, a 28 amino acid linear peptide, are proteolytically cleaved from the same 130 amino acid precursor protein, prepro-orexin (125). Within the CNS, orexin-containing neurons are found primarily in the LHA and the perifornical region (PeF) (126, 127). These neurons project widely to areas throughout the CNS including the locus coeruleus, raphé nuclei, periaqueductal central gray, PVN, ARC, the cortex, the thalamus, the lining of the third ventricle and the spinal cord (126-130). This projection pattern suggests that the OXA system regulates multiple physiological functions including feeding behavior, sleep states, neuroendocrine function, and autonomic control (126, 127, 131, 132).

Within the hypothalamus, orexin-containing neurons innervate a number of nuclei important in the regulation of feeding behavior including the preoptic area, PVN, LHA, DMN, VMN and the ARC (127,132-137). Orexin-containing fibers synapse on perikarya and dendrites in the ARC and also terminate in close apposition to NPY- or agouti-related protein-containing nerve terminals in the PVN (126, 127, 137).

Prepro-orexin mRNA expression in the LHA is increased by complete fasting (48 h) but not by food restriction (138, 139) suggesting activation of these neurons under conditions of hunger. Ghrelin, an appetite-stimulating hormone has recently been shown to activate OXA neurons (140). Fasting possibly leads to the activation of ghrelin, which is secreted from the stomach (141,142), and the blood levels of which increase with fasting and fall promptly after eating (141). Therefore ghrelin has been implicated as a physiological mediator of meal initiation.

Centrally administered ghrelin stimulates orexigenic activity through NPY and agouti-related protein (142). Ghrelin axonal terminals innervate and make direct synaptic contacts with orexin-producing neurons (141). ICV administration of ghrelin induced *fos* expression, a marker of neuronal activation, in orexin-producing neurons (143-145). Therefore evidence has been presented that show an interacting hypothalamic pathway between ghrelin and orexin in the regulation of feeding behavior and energy homeostasis (141).

The orexins act on two G protein-coupled receptors , orexin type 1 receptors (OX1R, HCRT-R1) and orexin type 2 receptors (OX2R, HCRT-R2) (138, 146-149). The highest concentrations of OX1R and OX2R are found in the VMH and PVN nuclei respectively, although several other hypothalamic and extrahypothalamic regions also contain high concentrations of OXA receptors (146-149). OX1R is considered to be a selective receptor for OXA as this receptor has a much higher affinity for OXA than for OXB (138,149) and OX2R is considered to be a non selective receptor with equal affinity for both OXA and OXB (138).

Distinct expression patterns of mRNA for both OXA receptors have been observed throughout the brain (150,151). OX1R mRNA is expressed in many brain regions including the prefrontal cortex, hippocampus, medial ventral thalamic nucleus, VMH, LHA, dorsal raphé nucleus, and locus coeruleus (146-149).

OX2R mRNA distribution is seen throughout the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and many hypothalamic nuclei such as the tuberomammillary nucleus, DMH, PVN and the ventral premammillary nucleus (150,151). The differential distribution of orexin receptors is consistent with the multifaceted roles of orexin in regulating endocrine homeostasis, automatic control and food regulation and energy homeostasis.

Central administration of OXA increases food intake by delaying the onset of a behaviorally normal satiety sequence (eating, grooming, resting) (152,153). Orexin A induced feeding has been demonstrated following ICV injections (153) or specific brain site injections in the perifornical hypothalamus LHA, PVN and the ventral tegmental area (152-155).

Some studies suggest that OXB plays little or no role in feeding behavior, or even that it may inhibit feeding (156,157). The greater effect of OXA compared with OXB appears to implicate OX1R rather than OX2R in the regulation of feeding. The distribution of OX2R is consistent with a role in sleep/wake states. There is a possibility that OXB has less effect because it is more rapidly cleared from the area of injection. Peripherally administered, OXB is known to metabolize faster than OXA (157).

Food deprivation increases OX1R and OX2R mRNA and protein levels in the hypothalamus (158), in addition to inducing a fivefold increase in prepro-OXA mRNA in the LHA (158). Prolonged fasting (48–72 h) and/or insulin-induced hypoglycemia have also been found to increase hypothalamic prepro-OXA mRNA expression, hypothalamic OX1R densities (158) and *c-fos* activation in LH OXA neurons.

However, investigation of the role of the orexins in feeding has been hampered by the lack of selective orexin receptor antagonists. The most potent and selective antagonist is the OX1R antagonist SB-334867, which has at least 50-fold higher affinity for OX1R vs. OX2R and penetrates the brain following systemic administration (159). SB-334867 suppresses food intake and advances the onset of a normal satiety sequence (160). It is increasingly being used as a tool to study OX1R responses to exogenously administered peptides and is also used to appraise the physiological and behavioral significance of endogenous orexins.

Overview of Research

Rationale

Orexin- containing neurons are morphologically linked with NPY-producing neurons in the hypothalamus. The purpose of this study is to find out more about the functional relationship between the two orexigenic peptides. Research conducted in this lab showed that simultaneous subthreshold doses of OXA and NPY agonists interact to stimulate feeding (unpublished data), and other studies have shown that NPY and OXA antagonists injected individually into the PVN and LHA, respectively, suppress NPY - and OXA- induced-food intake (cite).

Based on the evidence above, we hypothesize that there is an interdependence of NPY and orexin signaling in feeding behavior. More specifically, we hypothesize that slight reductions in the activity of orexin and NPY receptor bearing neurons, using simultaneous administration of subthreshold doses of receptor-specific antagonists, will inhibit feeding behavior. Thus we predict that simultaneous administration of subthreshold doses of NPY- and OXA-specific antagonists will significantly reduce feeding.

To test this hypothesis, in Experiment 1, we will first characterize OX1R antagonist SB 334867 and determine the lowest effective dose for inhibition of NPY induced feeding. In Experiment 2, we will determine the subthreshold dose of NPY Y1 receptor antagonist for deprivation-induced feeding in rats. Finally, in Experiment 3, we will test the effect of simultaneous administration of subthreshold doses of both antagonists.

If the predictions are accurate and the primary hypothesis is validated it will help further augment evidence for the existence of a functional link and between OXA and NPY- producing neurons in the LHA and the PVN, respectively, in the regulation of feeding.

Material and methods

Animals

Male Sprague–Dawley rats (275-350g, Charles River Laboratories, Wilmington, MA) were housed individually in stainless steel hanging cages with a 12-h light/dark cycle (lights on at 07:00 h) in a room at 21–22 °C. Rats were given continual access to standard rodent chow (Harlan Teklad, Madison, WI) and water was given *ad libitum*, except where noted. All efforts were made to minimize animal suffering and reduce the number of animals used in these studies, and all experiments received Institutional Animal Care and Use Committee approval at the Minneapolis Veterans Affairs Medical Center.

Surgeries and cannula placement

Rats were anesthetized with Nembutal (IP) (50 mg/kg body wt.) and fitted with either unilateral or bilateral 26 gauge stainless steel guide *cannulae* (Plastics One, Austin, TX) placed in the PVN and/in the rLHA. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (1998) and are as follows: rLHA: 1.9 mm lateral, 2.2 mm posterior to Bregma, 7.2 mm below the skull surface; PVN: 1.9 mm lateral, 0.5 mm from Bregma, 7.3 mm below the skull surface. The injector extended 1 mm beyond the end of the guide cannula. For all cannulations, the incisor bar was set at 3.3 mm below the ear bars. Dental cement was used to secure the cannulae to two screws inserted in the skull. Rats were allowed 1 week of postoperative recovery before initiation of experimental trials. Seven to 10 days elapsed between surgery and the first injection.

Drugs

Orexin Antagonist SB 334867 (Tocris Bioscience, Ellisville, Missouri, USA) and NPY (Phoenix Pharmaceuticals, Mountain View, CA) and NPY BIBP3226 (American Peptides, Sunnyvale, CA, USA) were dissolved in artificial cerebrospinal fluid (aCSF), aliquoted and kept at 4 °C until needed. All injections given to the control animals contained the vehicle corresponding to that used for the treated animals.

Injections

Injection cannulae (28 gauge; Plastics One, Roanoke, VA, USA) were directed to the PVN and the rLHA unilaterally or bilaterally in separate experiments. Dose volumes of 0.5 µl were injected slowly over 60 s to ensure proper diffusion and to minimize distribution of drug upwards on the cannula tract. After injection, the stylet was replaced and the rat returned to his hanging cage.

Food Intake Measurements

Food was allowed ad libitum until the start of each experimental trial. Just before injection, food was removed, and immediately after injection, pre-weighed pellets of chow were placed inside a steal hopper adhered to the hanging cage. At selected time points (1h, 2h. 4h), pellets and spillage were weighed and subtracted from the initial weight to quantify the amount of food eaten.

Food-deprivation regimen

Rats were food deprived for 24 hrs. After this period, pre-weighed hoppers were returned to home cages. Food intake was quantified and corrected for spillage after 1 h, 2 h and 4 h.

Verification of Placement

After both experiments, brains were dissected out and stored in a 10% formaldehyde solution for placement verification by histological examination. The brain tissues were sectioned with a cryostat at thickness of 50-μm, mounted on gelatin-coated slides, stained with 0.1% thiamin, and treated with ethanol (from 30% to 100%) and Clearing Agent (Electron Microscopy Sciences, Hatfield, PA). After the slides were dried, injection placement was determined microscopically at x10 magnification, using the brain atlas of Paxinos and Watson (1998) as a reference.

Statistical Analyses

The initial SB 334867dose response study data was analyzed using a two–factor ANOVA for repeated measures. Subsequent analysis was done using a one-factor ANOVA for the remaining experiments by GraphPad Prism (GraphPad Software, San Diego California USA).

Experiment I Orexin Antagonist SB 334867 Dose-Response

This dose-response study was conducted to replicate previous findings of dose response studies that showed the subthreshold dose for the orexin antagonist SB 334867 was 100pmol.

Methods

A total of adult male SD (n=30) rats were used in a randomized Latin square design (repeated measures). Rats were implanted with double contralateral guide *cannulae* directed at the PVN (AP: -1.9; Lat: ±0.5; D/V: -7.3) and rLHA (AP: -2.2; Lat: ±1.9; D/V: -7.2) respectively. Over a period of six days, the SD rats were randomly administered each of the three treatments as follows: Rats received (1) aCSF into both *cannulae*, (2) NPY alone (117 pmol in 0.5 µl aCSF) into the PVN and aCSF into the rLHA, or (3) NPY into the PVN paired with 10, 30, 100, or 300 pmol SB 334867 into the rLHA. After verification of cannula placement seven (8) data sets were excluded. Data was analyzed using two-way repeated measures ANOVA Statistical Analyses. For this specific experiment, data was analyzed using GraphPad Prism (GraphPad Software, San Diego California USA).

Results

The results showed that during 0-1 h food intake measurement, the only significant difference was observed for the 100 pmol dose of SB 334867 compared with the vehicle (Fig 1). In the 0-2 h time interval, the SB 334867 dose of 300 pmol showed significant differences comparatively with the vehicle (Fig 2). During the 0-4 h time

interval, none of the treatments specified caused significant differences in food intake compared with the vehicle (Fig 3).

Discussion

SB-334867 is increasingly being used as a tool to study OX1R responses to exogenously administered peptides and suppresses food intake. The objective of this experiment to determine the subthreshold dose of SB 334867 was obtained.

During this dose response study the initial studies were done with higher doses and our results showed inhibition of food intake for all treatments. After numerous trials we went to a lower dose and finally got the subthreshold dose that reduced food intake. During all time points of food intake measurements the effects of NPY administration in the PVN is apparent. SD rats receiving NPY immediately increased food intake.

During the first hour (1 h) food intake measurement the only dose of SB 334867 that showed significant differences was the 100 pmol dose. A discrepancy was noted because the 300 pmol dose did not reflect significant differences this being a more potent dose. During the second hour (2 h) of food intake measurements this changed and the 300 pmol dose showed significant differences comparatively with the vehicle. In contrast during this time point, the SB 334867 100 pmol dose showed no significant differences.

During the last time point (4 h) the orexin antagonists (10, 30, 100, 300 pmol) showed no significant differences whatsoever. The decision was made to abide by the 0-2 hour food intake measurement where it clearly shows that at 300 pmol was the least effective dose and where significant differences were achieved. Based on these results, we conclude that the subthreshold dose for the orexin antagonist SB 334867 is 100 pmol.

Experiment 2 BIBP 3226 Dose-Response – Food Deprivation

The objective of this study was to determine the subthreshold dose of NPY antagonist BIBP 3226 for a future dose-response study. The initial studies were done with higher doses and gradually a decrease in doses determined the lowest effective dose that inhibited food intake and the subthreshold dose for BIBP 3226. In this experiment, doses less than 0.75 nmol were tested to determine the subthreshold dose for BIBP 3226. The previous study suggests that any dose administered higher than or equal to 0.75 nmol should block food intake. If the dose range tested in this experiment still shows inhibition of intake, another dose-response study may be required.

Methods

A total of fifty-three (n=60) Sprague-Dawley (SD) rats were used for this study. SD rats were implanted with a single unilateral cannula directed at the PVN. Stereotaxic coordinates for the PVN (AP: -1.9 mm; Lat: ± .5 mm; D/V: -7.3 mm) were determined using the rat brain atlas of Paxinos and Watson (1998). SD rats were divided in three groups (n = 17, 18, and 18, respectively) corresponding to three days. The SD rats were then randomly divided into three groups per day (average six per group). Each individual SD rat was randomly administered either the vehicle or one of the doses over the course of three days.

SD rats were injected with artificial cerebrospinal fluid (aCSF) alone, or NPY antagonist BIBP 3226; (0.75, 0.6, 0.4 and 0.2 nmol respectively in 0.75 µl aCSF) following 24h food deprivation. After verification of cannula placement seven (7) data sets were excluded. One-way ANOVA was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

Results

During the first hour (1hr) food intake central administration of BIBP 3226 in the PVN reduced NPY-induced food intake in all doses tested (0.75, 0.6, 0.4 and 0.2 nmol) compared with vehicle (Fig 4). During the second hour (2h) and fourth hour (4h) food intake measurements significant differences were recorded for all doses except the 0.4 nmol dose (Fig 5, 6).

Discussion

There was some inconsistency in the results for animals receiving the 0.4 nmol dose of BIBP 3226. Central administration of the 0.4 nmol dose effectively inhibited food intake during the 1hr time period only, while a lower dose (0.2 nmol) effectively inhibited food intake during the 1h, 2h and 4h time frames raising doubts about the 0.4 nmol dose's efficiency and strength. It is possible that an error in dilution of the antagonist occurred when preparing this particular dose. Despite the potential concern over the 0.4 nmol dose, it is clear from this study that the subthreshold dose of BIBP 3226 is likely lower than 0.2 nmol.

Experiment 2A: BIBP 3226 Dose-Response II

Based on the results of Experiment 1A, it is clear that a subthreshold dose of BIBP 3226 is less than the lowest amount used in the previous study (0.2nmol). The previous study suggests that any dose administered higher than or equal to 0.2nmol should reduce food intake. Based on the significant effects of the lower doses in the 0-1h time frame of the previous study, we chose to repeat the study using picomolar amounts of the antagonist. Administration of the doses in picomoles should provide a dose small enough to determine the subthreshold dose of BIBP 3226 in these animals.

Methods

The same experimental design and methodology used in the preceding dose response studies were used. SD rats (N=53) were divided in three groups (n = 17, 18, and 18) as described in Experiment 1A. Treatments used in this study were similar to those used in Experiment 1A, except that BIBP 3226 doses were 25 pmol, 50 pmol, 100 pmol and 200 pmol respectively. Each individual SD rat was randomly administered either the vehicle or one of the doses over the course of three days. Measurements of food intake were restricted to only the first 1h interval following treatment. Data were analyzed using one-way ANOVA (GraphPad Software, San Diego California USA).

Results

The results in the first hour (1h) food intake measurements showed that the 200 pmol dose of BIBP 3226 was the lowest dose at which significant differences was observed when compared to vehicle (Figure 7).

These results indicate that the 200 pmol dose is the lowest effect dose. No significant differences were observed in any of the other treatments.

Discussion

With each successive dose- response study the gradual increase of food intake and the time for the food intake measurements to reach the previous established food intake baseline increased. The slow increase of food intake and the time taken to reach the baseline established beforehand was motive for concern. This raised the issue of the feasibility of using these rodents in another dose-response study.

One possible explanation for this delay in food intake response may be that administration of BIBP 3226 resulted in irreversible drug binding to the receptor. It may have caused irreversible change to the receptor or its capacity to respond and therefore removing its response potential from the system. Therefore it was determined to measure the first hour intake only.

The SD rodents were administered treatments ranging from 200 pmol to 25 pmol. Food intake response was similar across doses except for significant differences found in the 200 pmol dose. This result was evident after the 1h interval. It was determined that the 100 pmol dose is the subthreshold dose for BIBP3226.

Orexin and NPY Antagonist Subthreshold Experiment

Research conducted in this lab has provided evidence that feeding behavior stimulated by orexin in the LHA is mediated in part by interaction with NPY responsive pathways in the hypothalamic PVN. However, no studies to date have examined the behavioral response to simultaneous administration of subthreshold doses of both the NPY and orexin receptor antagonists. Here we tested this paradigm by measuring the food intake response to simultaneous central administration of subthreshold doses of the NPY receptor antagonist BIBP 3226 and the orexin 1 receptor antagonist SB 334867 on deprivation induced feeding, using the subthreshold doses of each antagonist identified in Experiments 1 and 2.

Methods:

A total of fifty-three (n=53) Sprague-Dawley (SD) rats were used for this study. Rats were implanted with double contralateral guide *cannulae* directed at the PVN (AP: -1.9; Lat: ±0.5; D/V: -7.3) and rLHA (AP: -2.2; Lat: ±1.9; D/V: -7.2) respectively. These coordinates were determined using the rat brain atlas of Paxinos and Watson (1998). SD rats were divided in three groups (n = 17, 18, and 18, respectively) corresponding to three days. The SD rats were then randomly divided into three groups per day (average six rats per group). Each individual SD rat was randomly administered either the vehicle or one of the doses over the course of three days.

SD rats were injected with artificial cerebrospinal fluid (aCSF) alone, or NPY antagonist BIBP 3226 100 pmol in the PVN, Orexin antagonist SB 334867 in the rLHA and both subthreshold doses of orexin and NPY administered simultaneously in the PVN and rLHA following 24h food deprivation. One-way ANOVA was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

Placement Verification

Histological examination of brain tissue to verify injection site showed that of the 53 SD rats only eighteen (n=18) were found to have correctly placed *cannulae*. Animals with incorrectly placed *cannulae* were excluded from the final analysis. Of the incorrectly placed cannula data sets, seventeen rats (n=17) were found to have *cannulae* misguided that were originally directed toward the rLHA and had been inserted directly into the nucleus accumbens (NAcc). The data from this specific data set was analyzed in an attempt to confirm specificity of effects regarding central administration of the orexin antagonist SB 334867 in the NAcc (Fig. 11 - 13).

Results:

Individual administration of subthreshold doses of SB 334867 or BIBP 3226 in the LHA and PVN respectively resulted in no significant inhibition of food intake. Significant differences were obtained when simultaneous administrations of both subthreshold doses were administered in comparison to vehicle (Fig 8, 9, 10). This was observed for all time points (0-1h, 0-2h, and 0-4 h).

Discussion:

Central food regulation in the CNS is modulated by a complex interconnected circuitry composed of populations of neurons that produce various orexigenic and anorexigenic neuropeptides, particularly in the hypothalamus. Due to the prevalence of obesity and its clinical implications it is imperative that we understand the physiology of food intake and the signaling mechanisms that modulate feeding behavior.

Studies investigating the control of food intake and energy expenditure have identified two specific neuropeptides, OXA and NPY, which affect food intake and are important in energy homeostasis. The present experiment investigated the interactions of OXA-containing neurons in the LHA and NPY-producing neurons in the PVN.

Previous studies have suggested interactions between orexin and NPY due to the abundance of axon terminals of orexin-containing neurons that terminate in the ARC and the PVN suggesting direct innervation of NPY-containing neurons by orexin fibers.

These data led us to believe that the orexin system may interact with the NPY system and serve as a stimulator of NPY-containing cells in the regulation of feeding behavior. This study sought to add more evidence to substantiate this premise by investigating the effect of simultaneous administration of subthreshold doses of the OXR1 antagonist SB 334867 in the LHA and the NPY Y1 antagonist BIBP 3226 in the PVN on deprivation-induced food intake.

A food deprivation paradigm was used here because deprivation-induced feeding should allow for a more innate physiological response concerning feeding behavior. The use of antagonists instead of stimulants allows for the collection of evidence of opposing physiological responses regarding orexin-NPY interactions. By combining these techniques we hoped to further substantiate evidence of orexin-NPY interactions in the control of food regulation.

Our prediction that simultaneous administration of antagonists would have an effect on intake at doses that proved ineffective when administered alone were accurate, and our data support the hypothesis that small changes in both NPY and OXA signaling are sufficient to affect food intake. This further supports evidence of a functional link between OXA- and NPY-producing neurons that cooperatively or reciprocally interact with each other to modulate feeding behavior.

Future Directions

Serendipity often plays a role in creating new avenues of interest that allow for the acquisition of further knowledge in the field of science. The misplaced *cannulae* originally intended for the rLHA and ending in the nucleus accumbens is an example (see Placement Verification). The evidence shows that simultaneous central administration of a subthreshold dose of an orexin antagonist in the nucleus accumbens (NAcc) in conjunction with a subthreshold dose of an NPY antagonist in the PVN reduced food intake (Fig 11 - 13). NAcc plays in regard to reward, motivation and reinforcement. Past research concerning the NAcc in this lab has shown evidence that the nucleus accumbens shell (AccSh) is a site of orexin modulation of feeding behavior and locomotor activity. This opens the possibility of interaction and modulation of feeding behavior by NPY in the NAcc via the PVN. More research concerning this should be forthcoming.

There are future experiments that are of interest to further elucidate orexin-NPY interactions. The ARC and the PVN have shown increased immunoreactivity for the early-active gene *c-Fos* after central administration of orexin. Conversely, *c-Fos* immunoreactivity has been observed in the LHA following central administration of NPY. The distribution of Y1 and Y5 receptors coincide with orexin receptor mRNA in the hypothalamus, notable in the ARC-PVN axis. Studies combining KO of individual or simultaneous receptors localized specifically in the PVN or rLHA in conjunction with central administration of either orexin or NPY in their specific target sites would be an interesting study. c-*Fos* immunoreactivity can then be measured after central administration in either the PVN or the rLHA.

Fig. 1 Dose- response of Orexin Antagonist SB 334867

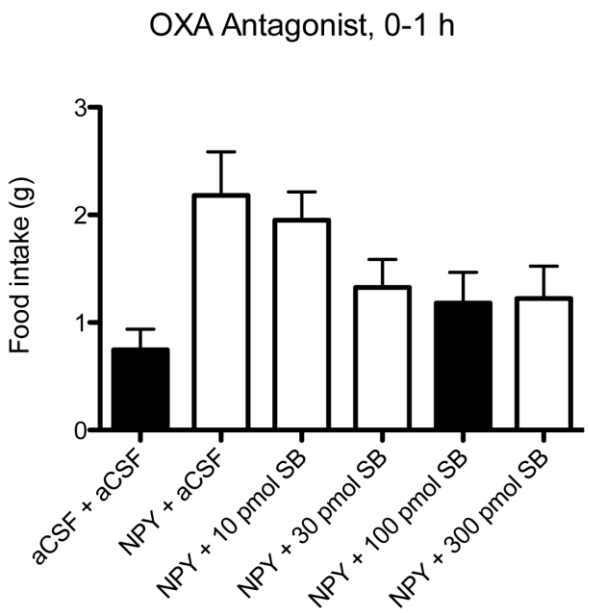
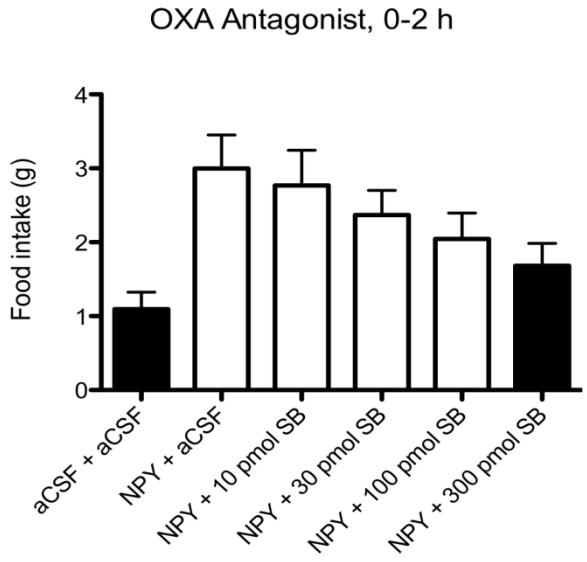
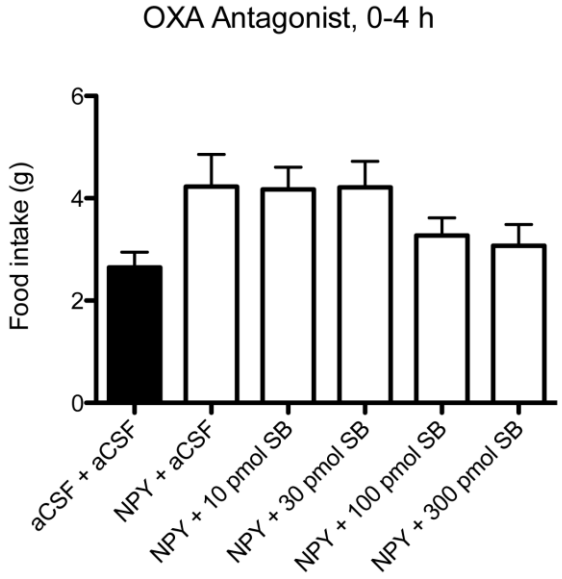


Fig. 2 Dose- response of Orexin Antagonist SB 334867



Dark bars indicate a significant difference from vehicle control ($p<0.05$, 22 per group) randomized Latin square design (repeated measures)

Fig. 3 Dose- response of Orexin Antagonist SB 334867



Dark bars indicate a significant difference from vehicle control ($p<0.05$, 22 per group) randomized Latin square design (repeated measures)

Fig. 4 Dose- response of BIBP 3226 (1)

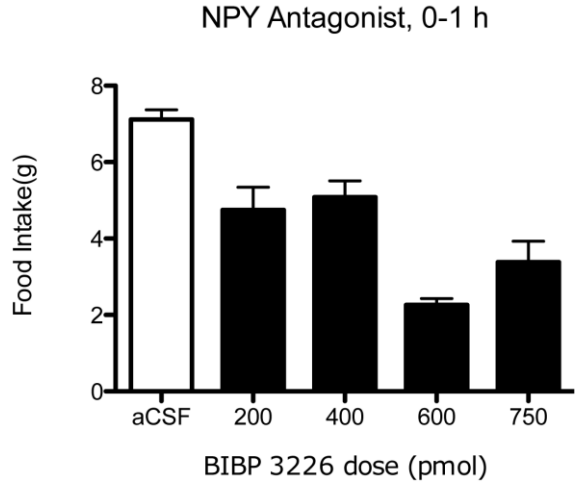
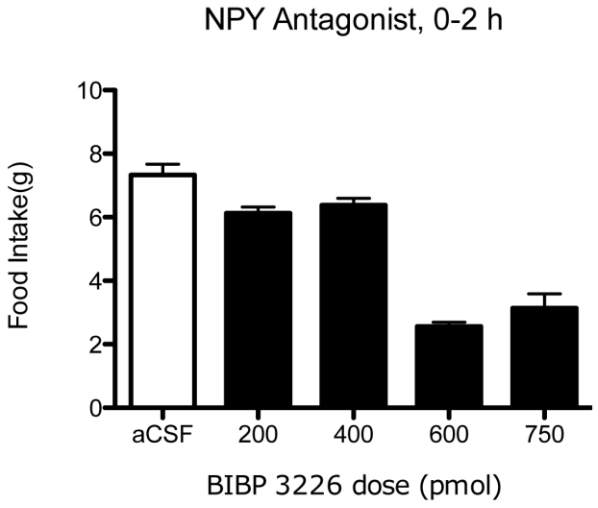


Figure 5 Dose- response of BIBP 3226 (1)



Dark bars indicate a significant difference from vehicle control ($p<0.05$, 7-9 per group) One- way ANOVA was performed

Figure 6 Dose- response of BIBP 3226 (1)

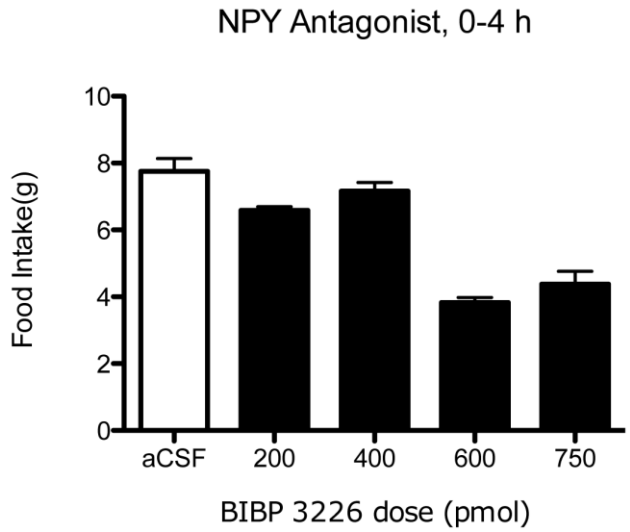
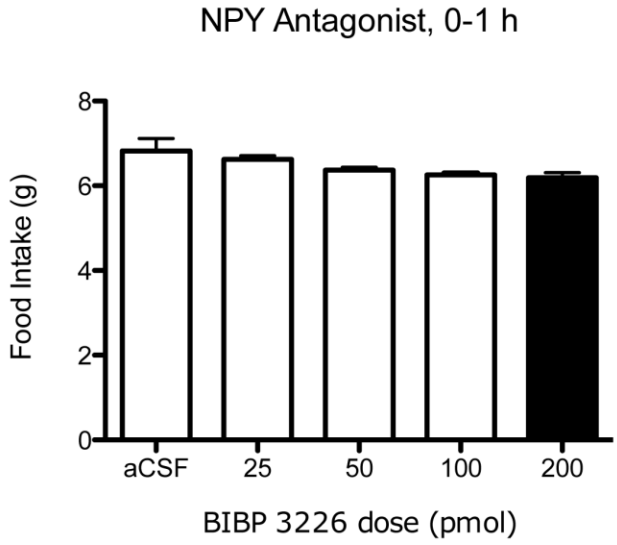
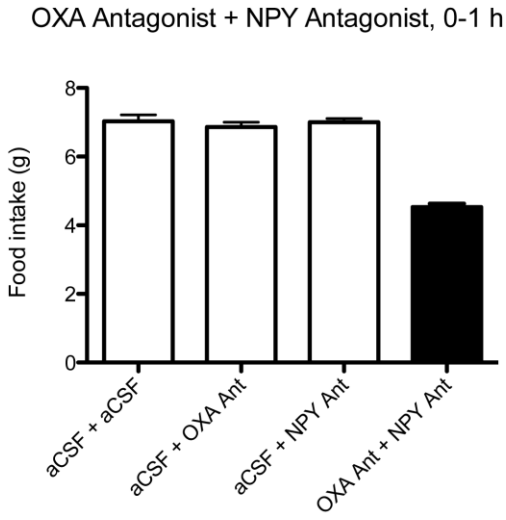


Fig. 7 Dose- response of BIBP 3226 (2)



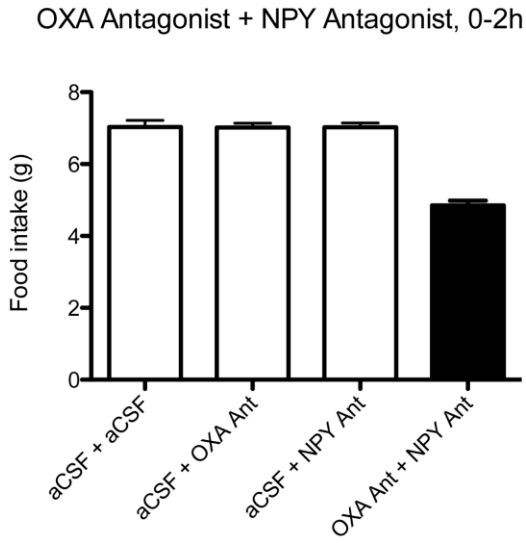
Dark bars indicate a significant difference from vehicle control ($p<0.05$, 7-9 per group) One- way ANOVA was performed

Fig 8 Orexin and NPY Antagonists' Subthreshold Experiment



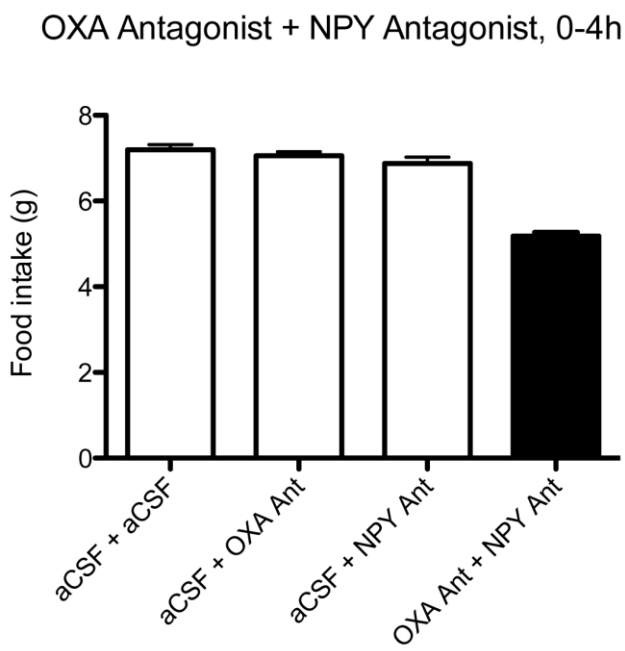
Dark bars indicate a significant difference from vehicle control ($p < 0.05$, n=18, 3-6 per group) One- way ANOVA was performed

Fig 9 Orexin and NPY Antagonists' Subthreshold Experiment



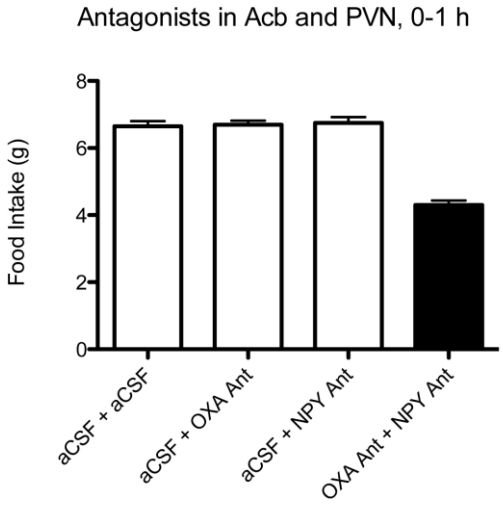
Dark bars indicate a significant difference from vehicle control ($p < 0.05$, n=18, 3-6 per group) One- way ANOVA was performed).

Fig 10 Orexin and NPY Antagonists' Subthreshold Experiment



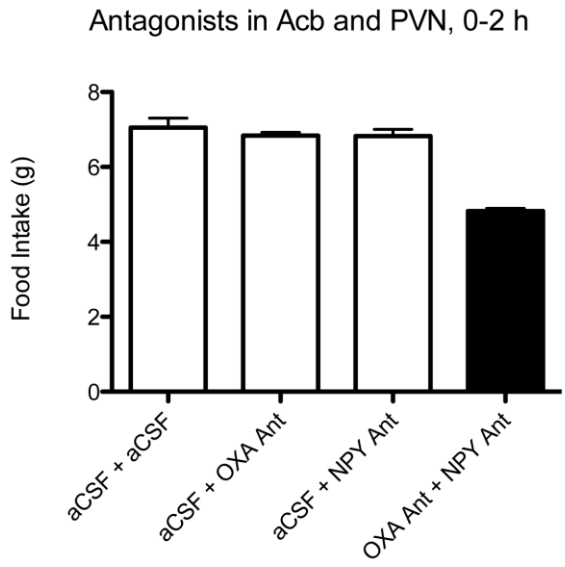
Dark bars indicate a significant difference from vehicle control ($p < 0.05$, n=18, 3-6 per group) One- way ANOVA was performed).

Fig 11 Nucleus Accumbens Subthreshold Dose-response study



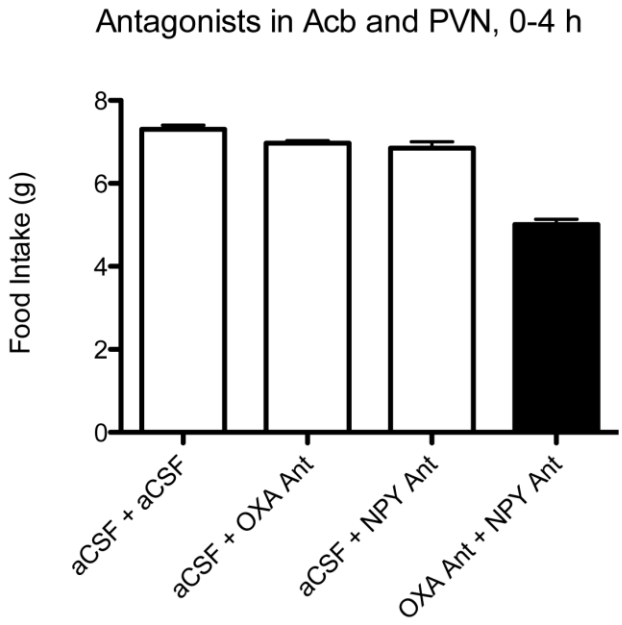
Dark bars indicate a significant difference from vehicle control ($p < 0.05$, n=17, 3-8 per group) One- way ANOVA was performed

Fig 12 Nucleus Accumbens Subthreshold Dose-response study



Dark bars indicate a significant difference from vehicle control ($p < 0.05$, n=17, 3-8 per group) One- way ANOVA was performed

Fig 13 Nucleus Accumbens Subthreshold Dose-response study



Dark bars indicate a significant difference from vehicle control ($p < 0.05$, n=17, 3-8 per group) One- way ANOVA was performed

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