DIFFERENTIAL EFFECTS OF THE KAPPA OPIOID RECEPTOR ON THE MELANOCORTIN ANTAGONIST AGOUTI-RELATED PROTEIN

by

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(Under the Direction of Silvia Giraudo)

ABSTRACT

This study investigated the effects of Nor-BNI co-administered and administered 24h after AgRP into the lateral ventricle (ICV) and the 3rd ventricle on AgRP induced feeding. Male Sprague Dawley rats were fitted with ICV or 3rd ventricle cannulas, then injected with 1nmol AgRP followed by 6.3nmol Nor-BNI co-administered or administered 24h after AgRP. Lateral ventricle Nor-BNI co-administered with AgRP significantly suppressed AgRP induced feeding at 24 h (p=0.0001). Lateral ventricle Nor-BNI administered 24h after AgRP showed no significant effect on AgRP induced feeding at any time point. 3rd ventricle Nor-BNI co-administered with AgRP significantly reduced sustained AgRP induced hyperphagia at 6, 24, 48, and 72h. 3rd ventricle Nor-BNI injected 24h after AgRP significantly reduced food intake at 48h after Nor-BNI injection compared to AgRP (p=0.026). These results along with prior research suggest that μ-opioid receptors may be involved in the prolonged feeding intake of AgRP in the 3rd ventricle.

INDEX WORDS: Melanocortin System, Opioids, Agouti-related protein (AgRP), Nor-Binaltorphimine (Nor-BNI), Food Intake
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CHAPTER 1
INTRODUCTION

Obesity is a large health problem in the United States as well as in the world. According to the Center for Disease Control and Prevention (CDC), 66% of American adults are either overweight or obese [1]. The prevalence of obesity is a major health concern due to its associations with several diseases including, but not limited to, type 2 diabetes mellitus, hypertension, osteoarthritis, dyslipidemia, coronary heart disease, heart failure, stroke, and cancers of the breasts, endometrium, prostate, and bowel [2, 3]. With the obesity epidemic becoming increasingly worse, it is important to look at why people are becoming obese. Obesity results from an imbalance of energy intake and energy output [4]. However, there are several contributing factors to the development of this imbalance, one of which is the neurological aspect of eating; In a world of plenty do we eat for pleasure and override the internal controls of feeding and body weight regulation? Many of the neural systems underlying these processes are not completely understood. Research has shown that two systems, the melanocortin and opioid systems play a role in the neurological regulation of feeding [5-8]. Melanocortins are bioactive peptides that are derived from the precursor molecule pro-opiomelanocortin (POMC) [3]. Of the five melanocortin receptors, melanocortin receptor 4 (MC4-R) and melanocortin receptor 3 (MC3-R) have been physiologically implicated in regulating food intake, body weight and energy homeostasis [9, 10]. The primary endogenous ligand for the MC3/4-R is alpha-melanocyte stimulating hormone (α-MSH). Data indicates that α-MSH/MC4-R plays an important inhibitory role in feeding and energy storage [7]. The Agouti-related protein (AgRP) is one of two naturally occurring endogenous antagonists of MC3/4-R. AgRP is a potent and selective antagonist of these melanocortin receptors [3]. Central
administration of AgRP in rodents can lead to increased food intake for up to one week [11]. An established method of AgRP action is competitive binding at MC3/4-R where AgRP inhibits signaling of α-MSH [12]. Studies suggest that at least part of the long-term affects of AgRP may be independent of its antagonist affects of MC3/4-R and may involve additional alternate mechanisms of action [3].

In addition to the melanocortin peptides, POMC cleavage also leads to the production of an opioid peptide, β-endorphin [13]. Opioid peptides increase food intake and are particularly effective at stimulating the consumption of palatable diets such as those high in sugar and other “attractive” tastants [14]. It is believed that opioid peptides provide the palatability and rewarding aspects of feeding rather than those for energy needs [3]. Comparisons have been made between the effects on feeding of AgRP and opioid receptor agonists [15]. AgRP seems to be involved in the maintenance rather than the initiation of feeding [16], and it was seen to have more influence on the intake of preferred versus non-preferred diets [17]. These results are similar to those observed in feeding following injection of opioids [6], [18].

The main objective of this study was to further investigate the antagonistic signal of AgRP to α-MSH/MC4-R and how it interacts with specific brain site opioid signals in the regulation of appetite and energy balance. Specifically, this study investigated the effects of Nor-Binaltorphamine (Nor-BNI), a κ-opioid receptor antagonist co-administered and administered 24 hours after AgRP into the hypothalamic and extra-hypothalamic area in the brain on AgRP induced feeding.

Current data indicate a significant amount of cross talk between melanocortin and opioidergic signaling in the central nervous system [12]. This research is significant because a clear and comprehensive understanding of this feeding-reward mechanism is far from complete.
Every new piece of evidence that contributes to our understanding of this overall puzzle is an important one, especially if we consider that this information could lead to the development of more effective weight management therapies to treat obesity and/or control appetite.
CHAPTER TWO

LITERATURE REVIEW

The Center for Disease Control and Prevention (CDC) states that 66% of Americans are overweight and that the death rate associated with obesity is higher than that caused by tobacco use [1]. In addition to increasing morbidity, obesity increases the risk for type 2 diabetes mellitus, hypertension, osteoarthritis, dyslipidemia, coronary heart disease, cardiovascular disease, heart failure, stroke, and cancers of the breasts, endometrium, prostate, and bowel cancers [2] [3]. Obesity results from an imbalance of energy intake and energy output [4]. This imbalance leads to an excess storage of body fat [3]. Appetite regulation is an important part of this equation because it determines the energy consumption or intake and consequent effect on body weight regulation. The control of body weight is a complex process that likely involves the interplay of numerous molecular mechanisms and neural circuits, many of them yet undefined. Feeding behavior is controlled by a series of short-term hormonal, psychological and neural signals derived from the gastrointestinal tract and the brain circuitry [3]. Other hormones such as insulin and leptin indicate long-term energy stores [3]. These signals and pathways all converge on the hypothalamus [3], which is thought to be a central region of feeding regulation [19]. There are also several extra-hypothalamic regions that play significant roles in feeding, particularly the nucleus tractus solitarius and area postrema in the brainstem, parts of the limbic system, the amygdala and the cerebral cortex.

A decade of research has highlighted the crucial role of brain circuitry in body weight regulation. Even though there is extensive evidence documenting the participation of the brain, genes and environment in the control of body weight, the neural systems underlying these processes
are not completely understood. Some of the most puzzling questions that remain to be answered are: Why/how do individuals become insensitive to the internal cues of satiety; What internal disruption of the biological systems occurs for an individual to reach obesity; In a world of plenty do we eat for pleasure and override the internal controls of feeding and body weight regulation? Identifying the mechanisms responsible for food reward, identifying the mechanisms responsible for hunger-related feeding, and identifying the interaction between the two might lead to new strategies to prevent overeating and could lead to more effective weight management therapies to treat obesity.

There are many neuroregulatory signals at particular brain sites that have been shown to participate in the regulation of food intake and could possibly influence each other. Some neuropeptides inhibit feeding; others seem to act as appetite stimulators, perhaps by altering energy signaling or by altering “rewarding” aspects of food [5, 16, 17, 20-22]. Two of these classes of neuroregulators, melanocortin and opioid peptides, are involved in a number of similar physiological processes such as analgesia and thermoregulation, where they typically have opposite effects [23-25]. Neurons and receptors from both systems often co-localize, and the opioid endorphins share a common precursor with melanocortins, pro-opiomelanocortin (POMC). Of the five melanocortin receptors, receptors 3 and 4 (MC3/4-R) have been physiologically implicated in regulating food intake, body weight and energy homeostasis [9, 10]. The primary endogenous ligand for MC4-R and MC3-R is alpha-melanocyte stimulation hormone (α-MSH), which is derived from POMC. When administered centrally, α-MSH inhibits food intake in rodents [26, 27]. Data suggest that α-MSH/MC4-R (rather than MC3-R) play an important inhibitory role in feeding and energy storage [7]. The agouti-related protein (AgRP) is one of two naturally occurring endogenous antagonists of MC3/4-R. In contrast to the effects of α-MSH, AgRP administered centrally
increases feeding with long lasting effects (longer than 24 hours) and is able to inhibit the actions of α-MSH [11]. An established method of AgRP action is competitive binding at MC3/4R where AgRP inhibits signaling of α-MSH [12]. Studies suggest that at least part of the long-term affects of AgRP may be independent of its antagonist affects of MC3/4R and may involve additional alternate mechanisms of action [3].

Where is MC4-R located in the brain?

MC4-R is expressed principally in the central nervous system and has been localized to a variety of sites in the rodent brain including the cortex, cerebellum, striatum, hippocampus, hypothalamus, midbrain, amygdala, thalamus and brainstem [13]. Consistent with its role in body weight regulation, the MC4-R is found in several regions of the brain thought to contribute to the regulation of feeding behavior. It is expressed in a number of hypothalamic sites, including lateral (LHA), dorsomedial (DMH) and paraventricular nucleus (PVN), which play important roles in regulating feeding behavior and energy metabolism [28]. These regions receive input from the hypothalamic arcuate nucleus (ARC) and the brainstem nucleus of the solitary tract (NTS), primary sites of AgRP and POMC expression in the brain [29, 30]. MC4-R mRNA is also expressed in several extra hypothalamic areas such a nucleus accumbens (Nac) and central amygdala (CeA) [31, 32]. These areas have also been shown to mediate opioid-related feeding [33]. It is reasonable to consider that these regions are important sites at which regulatory signals, such as those from the melanocortin and/or opioid systems are integrated. This suggests that the melanocortin system not only contributes to the homeostatic control of feeding but also to its food reward aspects via the MC4-R input to the Nac and CeA.
The opioid system on feeding

Exactly how opioids alter feeding is not known. A number of reports have defined opioids as orexigens which stimulate consumption through central reward-related mechanisms [14]. They increase the value of food by increasing its rewarding properties. Opioid receptors are present in virtually all central sites involved in the regulation of feeding. This includes the sites usually thought of as primarily energy-related, such as the hypothalamic paraventricular nucleus (PVN), as well as the sites associated with reward, such as the ventral tegmental area (VTA) [14]. The following findings substantiate this concept: 1) In humans, opioid receptor blockage (naloxone,) results in a decrease in the perceived pleasantness of sweet and salty foods and drinks [34]; 2) Naloxone more potently decreases consumption of palatable foods than non-palatable foods [18]; 3) Morphine increases intake of preferred food when the food is presented concurrently with a less preferred food [35]; 4) Consumption of palatable foods increases opioid binding and β-endorphin content in the hypothalamus of rats [36]; 5) Ingestion of palatable foods results in a naloxone reversible increase in nociceptive thresholds in rats [37] and 6) Threshold doses of naltrexone suppress feeding caused by neuropeptide Y (NPY) and AgRP [12, 38, 39]. It was also seen that opioid peptides stimulated ingestion of “attractive” diets in sated rats, enhanced the “dessert effect” when a palatable tastant was offered at the end of a regular meal, and made animals more motivated to get and eat palatable foods [14]. The rewarding aspects of consumption observed with opioids was a much greater effect than the effect opioids had on increasing the energy-driven aspect of food intake [14]. Hayward and colleagues also reported that mice lacking either enkephalin (a morphine like substances) or β-endorphin peptides showed a deficit in the ability of food reward to increase bar pressing behavior, regardless of palatability and nutrient content of the foods examined [40]. Kirkham et al. found that opioid receptor blockade altered meal maintenance without affecting
initiation, which is consistent with the idea that opioids affect the reward-related aspects of feeding rather than directly influencing the energy-related drive to eat [41].

Opioid agonists are also thought to delay satiety when consuming an “attractive” diet. It has been observed that rodents fed a “bland” diet terminate intake sooner than those presented with a sweet diet [14]. Levine observed that short-term food-deprived rats offered “unattractive” high-cornstarch diet eat approximately 30-50% less than animals given access to a high-sucrose diet [6]. These observations lead to the theory that satiety signaling is impaired or delayed when palatable foods are being ingested. This would allow foods to be consumed in the amount necessary to provide adequate sensory information rather than only to fulfill energy needs [42]. Conversely, opioid antagonists reduce the amount of consumed food and shorten the duration of a meal [42].

Studies have suggested that food rewards and drug rewards may share some common neural substrates, including substantial evidence that opioid receptors play key roles in both feeding and reward [43]. There are known neurochemical pathways that connect the “reward” nuclei such as the Nac and CeA with the “feeding” areas in the hypothalamus [38, 44, 45].

**Opioid receptors in the brain**

The opioid system has 3 receptor classes, but only the μ and κ receptors have been implicated in food intake [45]. The endogenous μ-agonists modulate reward, and blockade of their activity is aversive [46]. Evidence suggests that κ-agonists are not rewarding but facilitate food intake [47] by increasing taste “pleasure” [48, 49]. The opioid receptor antagonist of the μ and κ opioid receptors decreased feeding in animals and humans [50].

There are many sites throughout the brain where opioid receptors are found. These include several hypothalamic and extra-hypothalamic sites. The hypothalamus has received considerable
attention in studies investigating the role of opioids in feeding, and agonists of all opioid receptors subtypes stimulate food intake when injected into the PVN of the hypothalamus [14]. The κ-opioid receptors can be seen in many hypothalamic regions such as the arcuate nucleus, paraventricular nucleus, dorsomedial and ventromedial hypothalamus, and the lateral hypothalamic area [3]. These areas in the hypothalamus that are involved with feeding are typically involved with survival aspect of feeding, increasing food intake and decreasing energy expenditure when stores are depleted [3]. Therefore, these hypothalamic areas are more involved with the hunger aspect of feeding and not the rewarding aspect. The κ-opioid receptor can also be found in many extra-hypothalamic sites such as the nucleus accumbens, caudate-putamen, olfactory tubercle, bed nucleus stria terminalis, and the medial preoptic area [51]. There are several extra-hypothalamic regions that play significant roles in feeding, particularly the nucleus tractus solitarius and area postrema in the brainstem, parts of the limbic system, the amygdala and the cerebral cortex. Nuclei within the amygdala project to hypothalamic sites involved in feeding [47]. It was observed by Gosnell et al. that μ-opioid agonists injected directly into this area increased food intake, but κ-opioid antagonist infusion did not [47]. Data suggests that the amygdalar mu receptor could be important in the mediation of the orexigenic action of opioids [14].

**Agrp stimulation of feeding**

Several neuropeptides act as appetite stimulators, perhaps by altering energy needs or by altering “rewarding” aspects of food. Neuropeptide Y (NPY), opioid peptides, and AgRP increase food intake after central administration [5, 16, 20-22, 52]. AgRP is able to maintain an increase in food intake for up to one week [11]. AgRP is made in the arcuate nucleus of the hypothalamus in a population of neurons adjacent with neurons that produce α-MSH [53]. All of the AgRP-producing
neurons co-secrete NPY and project to various hypothalamic sites such as the PVN and dorsomedial hypothalamus (DMH), and project to extra hypothalamic sites [3]. AgRP is a potent and selective antagonist of MC3/4 receptors and is able to inhibit the actions of α-MSH [11]. An established method of AgRP action is competitive binding at MC3/4R where AgRP inhibits signaling of α-MSH [12]. Studies suggest that at least part of the long-term affects of AgRP may be independent of its antagonist affects of MC3/4R and may involve additional alternate mechanisms of action [3].

AgRP seems to have a more powerful influence on the intake of preferred versus non-preferred diets [17], and it appears to be involved in the maintenance rather than the initiation of feeding [16]. These effects are very similar to those observed following opioid receptor agonist injection [6, 54], [18]. Research coming from our laboratory, along with the studies of others in the field, suggests that opioid and melanocortin systems are functionally related in the regulation of food intake [39, 55-59].

How does Agrp maintain prolonged effects on feeding?

The prolonged appetite stimulation caused by AgRP has been and still is the major mystery of the melanocortin system. AgRP competitively binds to MC3/MC4-R, where it inhibits the action of α-MSH [53]. The characteristics of this response are long-lasting (several days). The acute, but not the long term hyperphagia caused by AgRP can be suppressed by MTII agonist to the MC3/MC4-R [59]. This implies that the feeding response caused by AgRP requires antagonism of MC-R but that the longer-term effects are due to activation of a signaling pathway downstream of the MC-R [53]. Data from our lab suggests that prolonged effects of AgRP may be due to the down-stream activation of opioid receptors. Present evidence suggests that AgRP not only contributes to the homeostatic control of feeding but also to its rewarding aspect, which would be
consistent with the involvement of the opioid system. Preliminary results show that naltrexone, a non-specific opioid receptor antagonist, given 24 hours after AgRP, can block AgRP prolonged feeding. This raises the possibility that AgRP is recruiting the opioid system to exert its long-term appetite stimulation. Feeding stimulation cause by AgRP is accompanied by a distinct pattern of c-Fos activation not only in hypothalamic nuclei but also in extra hypothalamic areas such as Nac and CeA [58]. This suggests that the melanocortin system might contribute to the reward aspects of feeding via the MC4-R input to the Nac and CeA. Is Agrp inhibiting satiation due to exaggeration of pleasurable aspects of food? At present the mechanisms by which AgRP stimulates prolonged feeding are not completely understood.

**Interactions between melanocortins and opioid peptides**

Data support the hypothesis that melanocortin and opioid peptides constitute a coordinated and balanced system modulating many physiological functions [60, 61], leading to the idea of an “opio-melanocortin homeostatic regulatory system” [62]. Though opioid and melanocortin peptides produce contrasting physiological effects, a large amount of evidence exists suggesting that the two systems are related. A number of studies have shown interactions between melanocortins and the μ-opioid receptor, and between β-endorphin and melanocortin receptors. Quillan and Sadee reported that dynorphin peptides selectively block α-MSH activation of the human MC3-R and MC4-R in vitro [63]. Unpublished results from our laboratory as well as recent publications by other colleagues have shown an interaction between the melanocortin and the κ and μ opioid peptides in the control of food intake when both are co-administrated [53, 57].

NPY and AgRP are co-localized on the same neurons. All of the AgRP-producing neurons co-secrete NPY [3]. A comparison of c-Fos like expression induce by AgRP and NPY suggests that
both acutely activate parallel neuroanatomic circuits [12]. It is considered that unknown mechanisms that are involved in AgRP-induced feeding may involve those underlying NPY-induced feeding [12]. An important central nervous system mediator of NPY-induced feeding and energy regulation is the opioid system [64]. It is thought because of this that opioid receptors may also play a key role in mediating AgRP’s effect on food intake [64].

This thesis addresses the action of the κ opioid receptor and its involvement in the interaction between the melanocortin and opioid systems.
CHAPTER 3

THE EFFECTS OF NOR-BNI CO-ADMINISTERED AND ADMINISTERED 24 HOURS AFTER AGRP INTO THE LATERAL VENTRICLE AND THE 3RD VENTRICLE ON AGRP INDUCED FEEDING

INTRODUCTION

Obesity results from an imbalance of energy intake and energy output [4]. However, there are several contributing factors to the development of this imbalance, one of which is the neurological aspect of eating. There are many neuroregulatory signals at particular brain sites that have been shown to participate in the regulation of food intake and could possibly influence each other. Some neuropeptides inhibit feeding; others seem to act as appetite stimulators, perhaps by altering energy signaling or by altering “rewarding” aspects of food [5, 16, 17, 20-22]. Many of the neural systems underlying these processes are not completely understood. Research has shown that two systems, the melanocortin and opioid systems play a role in the neurological regulation of feeding [5-8]. These two neuroregulators are involved in a number of similar physiological processes such as analgesia and thermoregulation, where they typically have opposite effects [23-25]. Neurons and receptors from both systems often co-localize, and the opioid endorphins share a common precursor with melanocortins, pro-opiomelanocortin (POMC). Of the five melanocortin receptors, receptors 3 and 4 (MC3/4-R) have been physiologically implicated in regulating food intake, body weight and energy homeostasis [7, 10]. The primary endogenous ligand for MC3/4-R is alpha-melanocyte stimulating hormone (α-MSH), which is derived from POMC. When administered centrally, α-MSH inhibits food intake in rodents [26, 27, 39]. Data suggest that α-
MSH/MC4-R (rather than MC3-R) play an important inhibitory role in feeding and energy storage [7]. The agouti-related protein (AgRP) is one of two naturally occurring endogenous antagonists of MC3/4-R. In contrast to the effects of α-MSH, AgRP administered centrally increases feeding with long lasting effects (longer than 24 hours) and is able to inhibit the actions of α-MSH [11]. An established method of AgRP action is competitive binding at MC3/4R where AgRP inhibits signaling of α-MSH [12]. Studies suggest that at least part of the long-term effects of AgRP may be independent of its antagonist affects of MC3/4R and may involve additional alternate mechanisms of action [3].

Data support the hypothesis that melanocortin and opioid peptides constitute a coordinated and balanced system modulating many physiological functions [56, 60, 61], leading to the idea of an “opio-melanocortin homeostatic regulatory system” [62]. Though opioid and melanocortin peptides produce contrasting physiological effects, a large amount of evidence exists suggesting that the two systems are related [56]. A number of studies have shown interactions between melanocortins and the μ-opioid receptor, and between β-endorphin and melanocortin receptors. Quillan and Sadee reported that dynorphin peptides selectively block α-MSH activation of the human MC3-R and MC4-R in vitro [63]. Unpublished results from our laboratory as well as recent publications by other colleagues have shown an interaction between the melanocortin and the κ and μ opioid peptides in the control of food intake when both are co-administrated [53, 56, 57].

Other studies have investigated the link between the AgRP induced feeding and opioid receptors. One study by Hagan et. al. investigated the effects of Naloxone (NALX), a non-specific opioid antagonist on AgRP induced feeding [12]. This study found that subthreshold doses of NALX administered into the 3rd ventricle blocked AgRP-induced intake when given simultaneously but not 24 hours after AgRP injection. However, Hagan’s study only looked at the injection of
NALX, the non-specific opioid antagonist into the 3rd ventricle, and only looked up to 24 hours after co-administration and up to 4 hours after NALX administration given 24 hours after AgRP on its effect on AgRP induced feeding. Our study differs in that we are looking at the specific κ-opioid receptor antagonist Nor-BNI in both the 3rd ventricle and the lateral ventricle. Our study also will observe the effects up to 120 hours after co-administration and observe the effect of Nor-BNI given 24 hours after AgRP up to 96 hours after Nor-BNI administration. Another study by Brugman et. al. investigated the individual and the combined effects of both the μ- and κ-opioid receptor antagonists and their effects on AgRP induced food intake [53]. This study focused on the short term effects, only up to 24 hours after administration into the 3rd ventricle, and also studied the effects of having a high fat diet versus chow. Our study differs from the study done by Brugman in that we are only looking at the effects on food intake of a chow diet, and feed intake will be monitored up to 120 hours after AgRP administration.

Current data indicate a significant amount of cross talk between melanocortin and opioidergic signaling in the central nervous system [12]. A clear and comprehensive understanding of this feeding-reward mechanism is far from complete. The goal of our experiments were to confirm the participation of the opioid and melanocortin systems in the regulation of food intake, and try to determine whether a selective κ-opioid receptor antagonist, Nor-Binaltorphimine (Nor-BNI) decreases food intake induced by AgRP when given in the hypothalamic region (3rd ventricle) and when administered in the extra hypothalamic region (lateral ventricle). This study specifically will look at the effects of Nor-BNI on AgRP induced feeding when co-administered and administered 24 hours after AgRP into the lateral ventricle and the 3rd ventricle.
MATERIALS AND METHODS

Animals and housing

Male Sprague-Dawley rats (Harlam, NC), weighting between 225 and 250 grams were housed in conventional hanging cages with a 12-hour light/12-hour dark photoperiod (lights on at 07:00 h) in a temperature-controlled room (21-22 °C). Rats had free access to standard chow (Purina Rodent Chow 5001: Purina Mills, MO) and water throughout the experiment. Animals were given a week to become accustomed to the environment before surgery.

Surgeries and cannula placement verification

Rats were anesthetized with a cocktail of ketamine (75 mg/kg) / xylazine (10 mg/kg) / atropine (10 mg/kg) at a dosage of 0.13 ml/100g body weight i.p. and fitted with 20-gauge stainless steel cannulas (Plastics One, Austin, TX). Stereotaxic coordinates for the lateral ventricle (ICV) were determined from the rat brain atlas by Paxinos and Watson [65] and were as follows: -1.0 mm posterior to bregma, 1.5 mm lateral to the midline, and -3.5 mm dorsal/ventral. Stereotaxic coordinates for the 3rd ventricle (3rdV) were determined also using the Paxinos and Watson rat brain atlas and were as follows: -1.5 mm posterior to bregma, 0.2 mm lateral from midline, and -1.5 mm dorsal/ventral. Dental cement was used to secure the cannula to two screws inserted in the skull. The injector extended 1 mm beyond the tip of the guide cannula. Seven days postoperative recovery was allowed before cannula placement was verified by administration of 5 μl injection of Angiotensin II (100 ng/5 μl sterile water). Rats that drank > 5 ml of water in the hour following Angiotensin administration were considered to have correct cannula placement and used for the experimental trials. Rats that did not drink > 5 ml in the hour following Angiotension administration were not used in the study and cannula placement was verified post mortem using
brain slicing verification. Data for rats whose cannula placement could not be verified were not 
used in the data analysis.

Following the cannula placement test, the correctly cannulated rats were transferred to the 
BioDAQ Food Intake Monitoring System (Research Diets, New Brunswick, NJ, USA) and allowed 
seven days to acclimatize before the beginning of experimental trials. The BioDAQ feeding system 
consists of twelve individual cages with weight sensing food hoppers wired to a central computer 
that continuously records food intake.

Drugs and injection

Injections were performed using Hamilton syringes (Hamilton, Reno, NV, USA). Rat 
AgRP was purchased from Peptides International (Louisville, KY, USA). Nor-Binaltorphimine 
(Nor-BNI) was purchased from TOCRIS (Ellisville, MO, USA). All injections were performed 
between 15:30 and 16:00 h, two hours before the beginning of dark cycle.

Feeding studies

Experiment 1: Dose response of Nor-BNI on AgRP induced food intake

The first 16 ICV cannulated rats were used in a dose response study to determine the lowest 
effective dose of Nor-BNI. Isotonic saline was used as a control solution. Agrp was administrated 
in a dose of 1nmol/ 5 μl. Based on previously studied doses [53], we tested 1.26 nmol (1 μg) and 
6.3 nmol (5 μg) of Nor-BNI. In random order the rats were assigned to one of 4 treatment groups. 
The treatment groups were: 1) saline + saline, 2) saline + AgRP, 3) Nor-BNI (1.26 nmol/ 5 μl) + 
AgRP, 4) Nor-BNI (6.3 nmol/ 5 μl) + AgRP. All of the treatments were delivered via cannula with 
fifteen minutes between the first injection and the last injection. Nor-BNI was administered before
AgRP. Food intake following the injections was recorded and analyzed at 24, 48, 72, 96, 120 and 144 hours (6 days). The food intake following the injection period was compared to an initial food intake average measured over a 4 day time period of normal intake.

Data were analyzed for food intake at 24, 48, 72, 96, 120, and 144 h by a one factor repeated measured ANOVA and means were compared using Fisher’s protected LSD test.

**Experiment 2:**

**Study 1: Effect of Nor-BNI co-administered with AgRP into the lateral ventricle on Agrp-induced food intake.**

Twenty four rats were ICV cannulated by the surgical procedure previously stated. In random order, the rats were placed into one of 3 treatment groups. The treatment groups were: 1) Saline + Saline, 2) Saline + AgRP (1nmol/5 μl), and 3) Nor-BNI (6.3 nmol/ 5 μl) + AgRP (1nmol/ 5 μl) Food intake following injections was monitored and analyzed at 1, 2, 4, 6, 24, 26, 28, 30,48, 72, 96, and 120 hours. Data were analyzed for food intake at 1, 2, 4, 6, 24, 26, 28, 30, 48, 72, 96, and 120 h by a one factor repeated measures ANOVA and means were compared using Fisher’s protected LSD test. Saline values were obtained from an initial food intake average measured over a 4 day time period of normal intake.

**Study 2: Effect of Nor-BNI administered 24 hours following AgRP into the lateral ventricle on Agrp-induced food intake.**

Twenty four rats were ICV cannulated by the surgical procedure previously stated. In random order, the rats were placed into one of 3 treatment groups. The treatment groups were: 1) Saline + Saline, 2) Saline + AgRP (1nmol/5 μl), and 3) AgRP (1nmol/ 5 μl) + Nor-BNI (6.3 nmol/ 5
μl) 24 hrs after. Food intake following injections was monitored and analyzed at 1, 2, 4, 6, 24, 26, 28, 30, 48, 72, 96, and 120 hours. Data were analyzed for food intake at 1, 2, 4, 6, 24, 26, 28, 30, 48, 72, 96, and 120 h by a one factor repeated measures ANOVA and means were compared using Fisher’s protected LSD test. Saline values were obtained from an initial food intake average measured over a 4 day time period of normal intake.

**Experiment 3:**

**Study 1: Effect of Nor-BNI co-administered with AgRP into the 3rd ventricle on Agrp-induced food intake.**

Twenty four rats were 3rd ventricle cannulated by the surgical procedure previously stated. In random order, the rats were placed into one of 3 treatment groups. The treatment groups were: 1) Saline + Saline, 2) Saline + AgRP (1nmol/5 μl), 3) Nor-BNI (6.3 nmol/5 μl) + AgRP (1nmol/5 μl). Food intake following injections was monitored and analyzed at 1, 2, 4, 6, 24, 48, 72, 96, and 120 hours. Data were analyzed for food intake at 1, 2, 4, 6, 24, 26, 28, 30, 48, 72, 96, and 120 h by a one factor repeated measures ANOVA and means were compared using Fisher’s protected LSD test. Saline values were obtained from an initial food intake average measured over a 4 day time period of normal intake.

**Study 2: Effect of Nor-BNI administered 24 hours following AgRP into the 3rd ventricle on Agrp-induced food intake.**

Twenty four rats were 3rd ventricle cannulated by the surgical procedure previously stated. In random order, the rats were placed into one of 3 treatment groups. The treatment groups were: 1) Saline + Saline, 2) Saline + AgRP (1nmol/5 μl), 3) Saline + Agrp (1nmol/5 μl) + Nor-BNI (6.3 nmol/5 μl).
nmol/5 μl) 24 hours after AgRP. Food intake following injections was monitored and analyzed at 1, 2, 4, 6, 24, 48, 72, 96, and 120 hours. Data were analyzed for food intake at 1, 2, 4, 6, 24, 26, 28, 30, 48, 72, 96, and 120 h by a one factor repeated measures ANOVA and means were compared using Fisher’s protected LSD test. Saline values were obtained from an initial food intake average measured over a 4 day time period of normal intake.

RESULTS

Experiment 1: Dose response of Nor-BNI on AgRP induced food intake

AgRP, administered at a 1 nmol dose significantly stimulated feed intake and maintained this effect up to 72 hours after administration (p = 0.01). Injection of Nor-BNI at a dose of 6.3 nmol (5 μg) / 5 μl significantly reduced intake of chow during the first 72 hours, compared with the intake of the AgRP treated group (23.8 +/- 0.872 v/s 21.5 +/- 0.303; p < 0.01) (Fig 1). This decrease in intake from the 6.3 nmol (5 μg) / 5 μl dose of Nor-BNI was not significantly different from the intake levels of the saline treatment group. Nor-BNI at a dose of 1.26 nmol (1 μg) / 5 μl had no effect on AgRP induced feeding at any time point (24, 48, 72, 96, 120, and 144 h). After 96 hours, all intake levels of treatment groups were equal. Intake remained unchanged between the treatment groups up to 144 hours. Because the 6.3 nmol (5 μg) dose of Nor-BNI did not decrease food intake to a level below the intake of the saline treatment group, but did suppress AgRP induced food intake, 6.3 nmol / 5 μl was the dose of Nor-BNI chosen for subsequent experiments.

Experiment 2:

Study 1: Effect of Nor-BNI co-administered with AgRP into the lateral ventricle on Agrp-induced food intake.
Rats receiving 1 nmol of AgRP significantly increased food intake above control levels and remained hyperphagic 24 hours after treatment (Fig 2) \((22.4 +/- .413 \text{ v/s } 27.45 +/- 1.05; p< 0.0001)\). Nor-BNI (6.3 nmol) co-administered with AgRP was able to significantly suppress AgRP stimulation of feeding bringing the food intake to control group levels at 24 hours \((p < 0.0001)\) (Fig 2). This decreased effect was not seen after 24 hours, indicating that the prolonged effect of AgRP is not blocked by Nor-BNI when co-administered in to the lateral ventricle.

**Study 2: Effect of Nor-BNI administered 24 hours following AgRP into the lateral ventricle on Agrp-induced food intake.**

Injection of Nor-BNI (6.3 nmol) administrated 24 hours after AgRP showed no significant effect on AgRP induced feed intake at any time point \((26, 28, 30, 48, 72, 96, \text{ and } 120 \text{ h})\). This indicates that the prolonged hyperphagia induced by AgRP is not decreased when Nor-BNI is injected 24 hours after AgRP administration in the lateral ventricle.

**Experiment 3:**

**Study 1: Effect of Nor-BNI co-administered with AgRP into the 3rd ventricle on Agrp-induced food intake.**

Rats receiving 1 nmol of AgRP significantly increased food intake above control levels and remained hyperphagic 24 hours after treatment (Fig 3) \((20.61 +/- .671 \text{ v/s } 25.96 +/- .823; p < 0.0001)\). Nor-BNI (6.3 nmol) co-administered with AgRP was able to significantly reduced the sustained AgRP-induced hyperphagia at 6, 24, 48, and 72 hours. The effect at 24 hours can be seen in Fig 3. After 96 hours the co-administered Nor-BNI no longer had a reducing effect on food intake.
Study 2: Effect of Nor-BNI administered 24 hours following AgRP into the 3rd ventricle on AgRP-induced food intake.

Nor-BNI (6.3 nmol) injected 24 hours after AgRP was seen to significantly reduce food intake at 48 hours after the Nor-BNI (6.3 nmol) was injected compared to AgRP intakes (Fig 4) (23.855 ± 1.8 v/s 19.965 ± 1.2; p < 0.026). This suppressing of feed intake was measured up to 96 hours. This will suggest Nor-BNI can reduce the prolonged AgRP induced hyperphagia when injected into the 3rd ventricle. At 120 h, all intakes were equal between the treatment groups.

DISCUSSION

The results from this study suggest that the κ-opioid receptor could be involved in the prolonged feeding effects of AgRP, but only in the 3rd ventricle. Nor-BNI significantly reduced AgRP induced feeding up to 72 hours when it was co-administered with AgRP in the 3rd ventricle. This could mean that the interaction on the melanocortin and the opioid systems is occurring in the hypothalamus. This would be expected since the hypothalamus is recognized as a central region of feeding regulation for energy needs [19]. However, when Nor-BNI was given 24 hours after the AgRP injection, it took an additional 48 hours to suppress intake. This effect was continued until 96 hours after AgRP administration.

These results were different then those observed by Hagan et. al. [12]. Hagan et. al. found that naloxone, the non-specific opioid antagonist did not suppress the prolonged effects of AgRP when co-administered and when given 24 hours after AgRP injected into the 3rd ventricle[12]. Hagan found that co-administration showed a decreased intake up to 3 hours after injection, but this effect disappeared at hour 4 and by 24 hours intake levels matched other treatment groups [12]. In Hagan’s study when looking at NALX injected 24 hours after AgRP, the effects were only observed
up to 4 hours after NALX administration, and NALX was seen to have no effect on reducing AgRP-induced hyperphagia [12]. This resulted in the conclusion that AgRP’s long-term effects did not depend on continued activation of opioid receptors [12]. Our study looked at the long-term effects of AgRP up to 120 hours after administration. This could be the reason that our study observed a long-term suppressing effect of Nor-BNI and Hagan’s study did not see this effect with NALX.

Brugman et al. also looked at the effects of specific opioid receptor antagonists on AgRP induced feeding [53]. This study focused on the short term effects, only up to 24 hours after administration into the 3rd ventricle. They found that Nor-BNI had no effect on AgRP’s orexigenic effect at 2 hours. They also found that β-FNA, the μ-opioid receptor antagonist had no effect on reducing AgRP induced feeding at any time point [53]. They did find that the combination of Nor-BNI and β-FNA significantly decreased AgRP induced intake of a high fat diet at 2 hours [53]. However, at 22 hours after injection, chow intake was not altered by the combined opioid receptor antagonists. This study did not look at the effects of the opioid antagonist beyond the 24 hour time point and they also used a high fat diet. Our study differed in that we observed intake up to 120 hours and we used a regular chow diet. The rewarding aspect of the high fat diet could have activated opioid receptors in extra-hypothalamic areas that are typically associated with reward. This could explain why Brugman et al. did not see a reducing effect from Nor-BNI in the 3rd ventricle (hypothalamic area). Our study only used regular chow and found that Nor-BNI significantly reduced AgRP induced feeding up to 72 hours when it was co-administered with AgRP in the 3rd ventricle.

The suppressing effect of Nor-BNI on AgRP induced food intake was not seen when Nor-BNI was injected into the lateral ventricle. This does not mean that the opioids in the lateral ventricle are not where the melanocortins and the opioid systems are interacting. Lateral ventricle
injections are targeted at extra-hypothalamic regions in the brain and there are several extra-
hypothalamic regions that play significant roles in feeding, particularly the nucleus tractus solitarius
and area postrema in the brainstem, parts of the limbic system, the amygdala and the cerebral
cortex. Extra-hypothalamic κ-opioid receptors binding sites include the nucleus accumbens,
caudate-putamen, olfactory tubercle, bed nucleus stria terminalis, and the medial preoptic area [51].
MC4-R mRNA is also expressed in several extra hypothalamic areas such as nucleus accumbens
(Nac) and central amygdala (CeA) [31, 32]. These areas have also been shown to mediate opioid-
related feeding [33]. The results seen in our study could be a result of the diet that was used in the
study, (Purina Rodent Chow 5001: Purina Mills, MO). Opioids are thought to provide the
palatability and rewarding aspects of feeding rather than those for energy needs [3]. They are also
thought to potentiate fat as well as protein ingestion [66]. With our study only using regular chow,
the rewarding aspects of the food may have not been activated. It is thought that opioids stimulate
ingestion of “attractive” diets, enhancing the “dessert effect” making animals more motivated to get
and eat palatable foods [14]. Our study may have not activated the rewarding aspect of feeding that
involves the extra-hypothalamic sites. Also, our study only tested the κ-opioid receptor in the
lateral ventricle. Gosnell et al. stated that μ-opioid agonists injected directly into the amygdala
increased food intake, but κ-opioid antagonist infusion did not [47]. The results seen could indicate
that another opioid receptor, other than the κ-opioid receptor is involved in the rewarding aspect of
AgRP induced feeding.

The hypothalamus is considered as a central region of feeding regulation for energy needs
[19]. It is not surprising that administration of Nor-BNI into the 3rd ventricle significantly
suppressed AgRP inducing feeding because of the M3 receptors and κ-opioid receptors that are
located there. The κ-opioid receptor can be seen in many hypothalamic regions such as the arcuate
nucleus, paraventricular nucleus, dorsomedial and ventromedial hypothalamus, and the lateral hypothalamic area [3]. The M3-R can also be found in a number of hypothalamic areas such as lateral (LHA), dorsomedial (DMH) and paraventricular nucleus (PVN), which play important roles in regulating feeding behavior and energy metabolism [28]. These areas in the hypothalamus that are involved with feeding are typically involved with survival aspect of feeding. The most powerful of the pathways involved with feeding that converge on the hypothalamus are those that increase food intake and decrease energy expenditure when stores are depleted [3]. Therefore, these hypothalamic areas are more involved with hunger and not reward, which could be the reason for the results seen in the 3rd ventricle portion of our study.

In summary, the neurological pathways involved with feeding are very complex and little is known about the involvement between the pathways. The neuropeptidergic pathways are part of a complex, dynamic network, whose balance reflects and defines the feeding activity of animals [14]. This study only investigated a very small part of the possible interactions of the opioid and the melanocortin pathways. One limitation of the study was the use of only regular chow. It would be important to investigate the effects of Nor-BNI administration on prolonged AgRP induced feeding in the lateral ventricle when different types of food are provided such as a high fat and a high sugar diet. Also, when investigating the melanocortin system one must also consider the effect of α-MSH, the endogenous ligand of MC3/4-R. The stimulatory effect of AgRP is inhibited by α-MSH [11]. Therefore, one must ask if the effects that are seen by the administration of Nor-BNI are not a suppression of AgRP but an activation of α-MSH.

Current data indicate significant cross talk between the melanocortin and opioid systems. However, there is much more research needed before the exact linkage between the systems is completely understood.
Figure 1: Effects of Nor-BNI at different doses on AgRP induced feeding at 24 hours administered in the lateral ventricle (ICV). Rats were first injected with either Nor-BNI at a dose of 1.23 nmol (1 μg) or 6.3 nmol (5 μg), or saline followed by 1 nmol AgRP. Saline injected rats served as controls. Values represented means ± SEM of 72 hour food intake. Values with different superscripts are significantly different (P < 0.01).
Figure 2: Effects of Nor-BNI co-administered with AgRP in the lateral ventricle on AgRP induced feeding 24 hours after administration. Animals were injected first with saline or 6.3 nmol Nor-BNI followed by 1 nmol AgRP. Saline injected rats served as controls. Values represented means ± SEM of food intake at 24 hours after AgRP injection. Values with different superscripts are significantly different (P < 0.0001).
Figure 3: Effects of Nor-BNI co-administered with AgRP in the 3rd ventricle on AgRP induced feeding 24 hours after administration. Animals were injected first with saline or 6.3 nmol Nor-BNI followed by 1 nmol AgRP. Saline injected rats served as controls. Values represented means ± SEM of food intake at 24 hours after AgRP injection. Values with different superscripts are significantly different (P < 0.0001).
Figure 4: Effects of Nor-BNI administered 24 hours after AgRP in the 3\textsuperscript{rd} ventricle on AgRP induced feeding 72 hours after administration. Rats were injected with AgRP at a dose of 1 nmol / 5 μl followed 24 hours later by an injection of either saline or Nor-BNI at a dose of 6.3 nmol / 5 μl. Values represented means ± SEM of food intake at 72 hours after AgRP injection. Values with different superscripts are significantly different (P < 0.026).
CHAPTER 4

SUMMARY

There are many neuroregulatory signals at particular brain sites that have been shown to participate in the regulation of food intake and could possibly influence each other. The goal of our experiments was to confirm the participation of the opioid and melanocortin systems in the regulation of food intake, and try to determine whether a selective κ-opioid receptor antagonist, Nor-Binaltorphamine (Nor-BNI) decreases food intake induced by AgRP when given in the hypothalamic region (3rd ventricle) and when administered in the extra hypothalamic region (lateral ventricle). Male Sprague Dawley rats were fitted with ICV or 3rd ventricle cannulas, then injected with 1nmol AgRP followed by 6.3nmol Nor-BNI co-administrated or administered 24h after AgRP. Food intake was measured utilizing the BioDAQ Automated Feeding System (Research Diets, Inc. NJ). Lateral ventricle Nor-BNI co-administered with AgRP significantly suppressed AgRP induced feeding at 24 h (p=0.0001). 3rd ventricle Nor-BNI co-administered with AgRP significantly reduced sustained AgRP induced hyperphagia at 6, 24, 48, and 72h. Lateral ventricle Nor-BNI administrated 24h after AgRP showed no significant effect on AgRP induced feeding at any time point. 3rd ventricle Nor-BNI injected 24h after AgRP significantly reduced food intake at 48h after Nor-BNI injection compared to AgRP (p=0.026). The results from this study suggest that the κ-opioid receptor could be involved in the prolonged feeding effects of AgRP, but only in the 3rd ventricle. More research is needed before the exact linkage between the systems is completely understood.
REFERENCES


