STUDIES ON THE MECHANISM OF ACTION OF GUT PEPTIDE PYY_{3-36}

by

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ABSTRACT

Peptide YY (PYY) is a hormone released from the gut in response to a meal. Peripheral administration of one form of the peptide, PYY_{3-36}, produces a short-term reduction in food intake in rodents. This peptide has achieved additional attention due to the observation that it reduces 24 h food intake and hunger scores in a small number of lean and obese human subjects, suggesting a potential clinical utility in the treatment of obesity. Initial reports suggested that effects of PYY_{3-36} on food intake are mediated by increasing the anorexigenic drive from melanocortin neurons in the hypothalamic arcuate nucleus. However, further understanding of the physiology of PYY_{3-36} was complicated, initially, by the fact that 12 laboratories had difficulty demonstrating that the peptide inhibits feeding in rodents. In this thesis work we demonstrate that the central melanocortin system does not mediate the anorexigenic actions of peripheral PYY_{3-36}; therefore, an alternative mechanism should be considered. We hypothesized that like other gut released peptides, PYY_{3-36} may mediate its anorexigenic actions through the dorsal vagal complex in the brainstem. Peripheral administration of PYY_{3-36}, at doses that reliably and reproducibly inhibit food intake, causes conditioned taste version and activates neurons in the area postrema, a circumventricular organ, and the intermediate nucleus tractus solitarius of the brainstem. Thus, inhibition of food intake by PYY_{3-36} may result in part from induction of an aversive response in mice. Further studies are needed to establish whether or not PYY_{3-36} could be an effective clinical tool in the treatment of obesity.

INTRODUCTION

Obesity

The Surgeon General of the United States Department of Health and Human Services recently declared obesity as one of the top five health priorities in the USA (Satcher, 2001). This epidemic is not limited to only the USA but now affects many nations; according to the World Health Organization (WHO) more than 1.2 billion people in the world are overweight (Puska P., 2003). Of particular concern is the rise in obesity in children in the US as well as in Europe and Asia (2005).

Obesity is defined as a body mass index (BMI) of over 30 (kg/m^2), while an individual with BMI of over 25 (kg/m^2) is considered overweight. Being overweight or obese is linked to extensive co-morbidity and mortality. Obesity is a risk factor for a variety of conditions, like type II diabetes, heart disease, stroke, arthritis, breathing problems, psychological disorders, like depression, and some types of cancer, including breast and colon cancer. In fact the incidence of type-II diabetes, traditionally known as "adult onset diabetes", in childhood has risen around 10 fold, such that it is now seen as commonly as type I diabetes. In the year 2000 in the US, the growing epidemic of obesity accounted for a total of about $117 billion (direct and indirect) healthcare dollars (Satcher, 2001).

The contributing causes of obesity are both environmental and genetic. Environmental factors include, but are not limited to, lifestyle (caloric intake and expenditure) and socioeconomic status. Obesity is often seen to be familial in its etiology. A multi-

disciplinary scientific approach is essential to elucidate the contributing causes of obesity, from the basic homeostatic systems involved in energy homeostasis to the complex environmental factors.

Identification of Hypothalamic Nuclei Involved in Energy Homeostasis

The obesity epidemic has heightened the need to understand the mechanisms underlying the control of body weight. The first indication that body composition and weight may be centrally controlled came in the late 1800's and early 1900's. Frohlich syndrome, caused by pituitary tumors, is associated with excessive subcutaneous fat deposition and hypogonadism (Bramwell, 1888; Frohlich, 1901). However, it was not until the 1940's and later in the 1970's, with the use of bilateral stereotaxic electrolytic lesions, that specific hypothalamic nuclei were implicated in the regulation of energy homeostasis (Anand and Brobeck, 1951a; Anand and Brobeck, 1951b; Hetherington and Ranson, 1940; Saper et al., 1976; Stevenson and Franklin, 1970; Swanson and Sawchenko, 1980). In particular, the dorsomedial hypothalamus (DMH), the ventromedial hypothalamic nucleus (VMH), the arcuate nucleus (ARC), the fornix, the lateral hypothalamus (LH), and the paraventricular nucleus (PVN), when ablated, were described to produce a distorted metabolic phenotype. Some nuclear lesions, e.g. PVN, were found to cause profound hyperphagia leading to obesity (Saper et al., 1976:Swanson, 1980 #4565), while others, like the LH lesions, caused loss of feeding, and even death by starvation (Anand and Brobeck, 1951a; Anand and Brobeck, 1951b). The LH lesions were later shown to interrupt the ascending nigrostriatal bundle, causing a Parkinsonian syndrome, typified by

the reduction of all voluntary movement, including feeding behaviors (Stricker and Verbalis, 1990). It was postulated that the hyperphagia associated with PVN lesions may result from the disruption of the autonomic control of gastrointestinal (GI) tract function, coming from either parasympathetic or sympathetic preganglionic neurons. Indeed, vagotomy prior to PVN lesion reverses the associated hyperphagia and resulting obesity (Cox and Powley, 1981). The PVN has reciprocal autonomic connections with nuclei within the dorsal vagal complex (DVC), like the nucleus tractus solitarius (NTS), which integrate visceral information and modulate satiety (Grill and Norgren, 1978b; McCann and Rogers, 1992; Zhang et al., 1998). However, sites other than the hypothalamus have been implicated in the regulation of energy homeostasis. With the development of a chronic decerebrate rat model (Flynn and Grill, 1988; Grill and Norgren, 1978a) in which connectivity between the forebrain and brainstem is severed, the brainstem has been shown to be sufficient to mediate responses involving taste and meal size, responding to variations in taste concentrations, gastric preload, cholecystokinin (CCK), and sham feeding (Grill and Kaplan, 1992; Grill and Smith, 1988; Seeley et al., 1994).

Molecular Mediators of Orexigenic and Anorexigenic Drive

The Central Neuropeptide Tyrosine (NPY) orexigenic system and receptors

While many neurotransmitters play a role in regulating energy homeostasis, this thesis is focused on the pancreatic polypeptide (PP) family. The PP family of peptides consists of neuropeptide Y (NPY), PP and peptide tyrosine tyrosine (PYY). All peptides in the

family have extensive sequence identity and code for a 36 amino acid long peptide with a caroboxyterminal amidation. However, each member has a unique distribution, resulting in diverse expression and function. PP and PYY are predominantly found in the periphery, where they are postprandially released and act as paracrine or endocrine hormones regulating gastrointestinal (GI) secretions and motility. NPY, on the other hand, is a neuromodulatory peptide found in neurons, both in the central nervous system (CNS) and peripheral nervous system (PNS) (Adrian et al., 1985; Bottcher et al., 1986; Bottcher et al., 1993).

NPY and PYY are the most potent orexigenic peptides ever tested following central administration (Clark et al., 1984; Stanley and Leibowitz, 1984), when compared to numerous other neurotransmitters and neuromodulators shown to cause mild hyperphagia, such as growth hormone releasing hormone (GHRH), γ-amino butyric acid (GABA), and the monoamines. In early work by Clark and colleagues, examining the effects of luteinizing-hormone, central administration of human PP was unexpectedly shown to stimulate food intake in rats (Clark et al., 1984). Clark and others subsequently showed that third and forth ventricular injections of NPY and PYY caused rats to consume substantial amounts of food, even when satiated (Clark et al., 1984; Stanley and Leibowitz, 1984). Following chronic central administration, NPY causes obesity, and produces many hormonal and metabolic changes, including hyperphagia, hyperinsulinemia, and lipogenic activity in liver and adipose tissue (Vettor et al., 1994; Zarjevski et al., 1993; Zarjevski et al., 1994).

Neuroanatomical studies have identified NPY as the most abundant peptide/neurotransmitter in both the CNS and PNS (Adrian et al., 1985; Bottcher et al., 1986; Bottcher et al., 1993). In the brain, NPY is expressed at high levels (Adrian et al., 1983; Allen et al., 1983) in sites shown, by bilateral stereotaxic electrolytic lesion studies and decerebration, to regulate energy homeostasis, such as the ARC, the DMH (Abe et al., 1990; de Quidt and Emson, 1986), and the NTS of the brainstem (Pickel et al., 1989). Gene expression and genetic studies have shown that NPY levels reflect orexigenic drive. Fasting induces large increases in NPY mRNA expression, and subsequent secretion, in the ARC (Broberger et al., 1998; Chua et al., 1991; Hahn et al., 1999; Korner et al., 2001; Sanacora et al., 1990) and PVN (Calza et al., 1989; Kalra et al., 1991). Furthermore, Takahashi and Cone used an electrophysiological slice preparation of the ARC to demonstrate that fasting produces a 4-fold increase in basal firing of NPY neurons (Takahashi and Cone, 2005).

The production of an altered body weight phenotype or change in feeding behavior resulting from the loss of a peptide or neurotransmitter is suggestive evidence for its importance in the regulation of energy homeostasis. Surprisingly, NPY knockout mice ($NPY^{-/-}$) have no obvious phenotype, and have normal food intake, body weight and adiposity (Erickson et al., 1996b). However, under more careful examination, a decreased re-feeding response to 24 and 48 h fasting was demonstrated (Bannon et al., 2000), consistent with the reduction of orexigenic drive. Also, crossing mice lacking the adipostatic factor, leptin (Lep^{ob}/Lep^{ob}) on to the $NPY^{-/-}$ background, partially reverses the obese phenotype of Lep^{ob}/Lep^{ob} mice, reducing the hyperphagia, diabetes, sterility, and

somatotropic defects while increasing energy expenditure (Erickson et al., 1996a). These data demonstrate that a significant component of the obesity syndrome in Lep^{ob}/Lep^{ob} mice results from chronic overexpression of NPY in the ARC nucleus.

All biological activities of NPY, and the other members of the family, are mediated via the NPY 1-5 (Y1-Y5) receptors (for review see (Gehlert, 1998; Hazelwood, 1993)), which recognize a common PP fold motif shared between all PP family peptides. Even though Y1-5 receptors all couple to G_i or G_o to inhibit cyclic AMP production, each subtype has a unique set of functions that result from their relative distribution (e.g. presynaptic vs. postsynaptic). Y5 and Y1 receptors are post-synaptically expressed on neurons in the PVN of the hypothalamus (Fetissov et al., 2004a; Fetissov et al., 2004b; Kishi et al., 2005; Parker and Herzog, 1999), as well as many other regions (Fig. 1), and mediate the orexigenic actions of centrally administered NPY (Inui, 1999; Mullins et al., 2001; Parker et al., 2000). Furthermore, Y5 receptor knockout mice ($Y5\text{-}R^{-/-}$) show an attenuated orexigenic response to central of NPY administration, which can be completely blocked by the administration of Y1 receptor antagonist (Pedrazzini et al., 1998). Additionally, direct intranuclear injection of NPY or PYY into the PVN causes profound hyperphagia (Stanley and Leibowitz, 1985) and intra-PVN injections of either Y1 or Y5 specific antagonists reverses c-Fos induction, and much of the orexigenic effect of centrally administered NPY (Wieland et al., 1998; Yokosuka et al., 2001; Yokosuka et al., 1999). Observations such as these, and others, suggest that the orexigenic effects of NPY may be partially mediated by the PVN.

Y1 Y2 Y4 Y5

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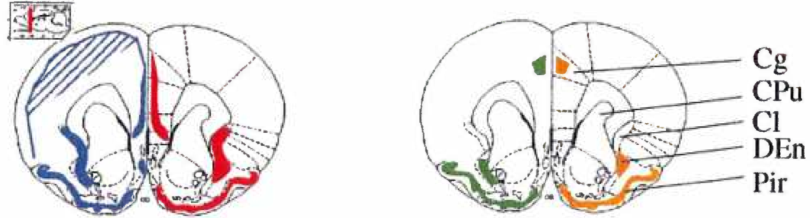

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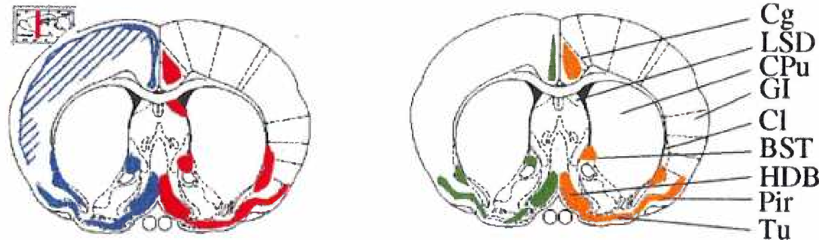

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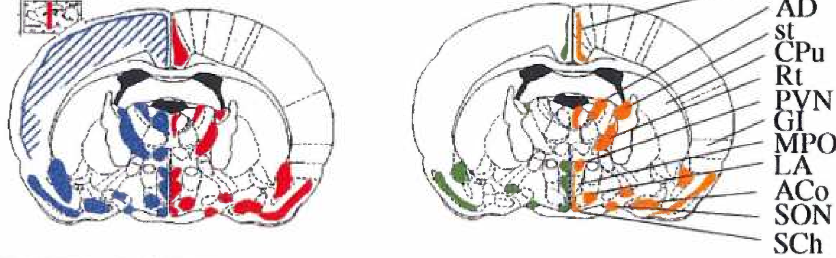

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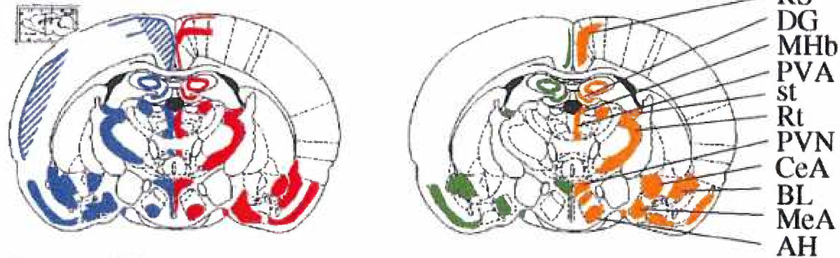

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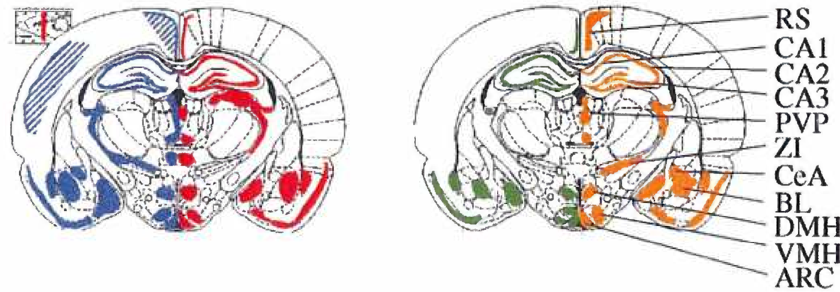

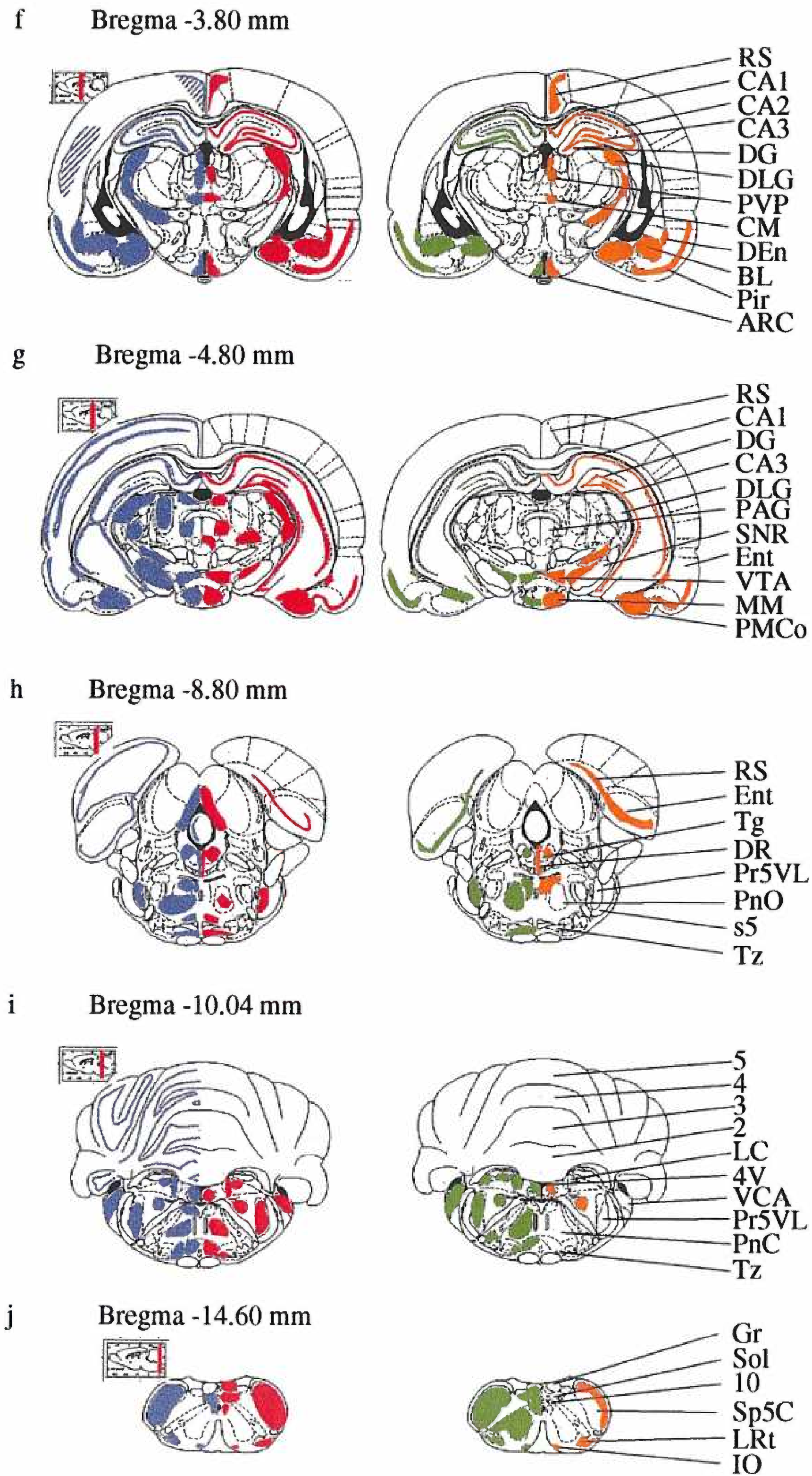

Fig. 1. Schematic diagram of serial coronal sections showing the distribution pattern of mRNA of Y1, Y2, Y4 and Y5 receptor subtypes in the rat brain. Position in relation to bregma is displayed on the top left. (Paxinos and Watson, 1997)

The Y2 receptor, on the other hand, is predominantly expressed on presynaptic terminals and may be involved in the modulation of NPY release (Broberger et al., 1997; Cowley et al., 2001b). Y2 receptor mRNA is widely distributed throughout the brain with high density of expression in the hippocampus and in centers implicated in the regulation of energy homeostasis, like the ARC, PVN and the NTS (Broberger et al., 1997; Parker and Herzog, 1999) (Fig. 1). Immunocytochemistry studies have identified Y2 receptors on presynaptic terminals of ARC NPY neurons (Broberger et al., 1997) and their activation leads to the inhibition of NPY release from NPY neurons (Cowley et al., 2001b; King et al., 1999). Site-specific administration of NPY_{13-36}, a Y2 specific agonist, into the PVN inhibits nocturnal food intake, especially the carbohydrate component of a meal (Leibowitz and Alexander, 1991). Also, Y2 receptor knockout mice develop mild obesity with increased fat deposition caused by hyperphagia and reduced energy expenditure (Naveilhan et al., 1999), further implicating the Y2 receptor as an NPY "anorexigenic" receptor. Intriguingly, the NTS, a major satiety center in the brainstem receiving vagal inputs from the gut, expresses high levels of Y2 receptor mRNA, but has very low levels of Y1 and Y5 receptor mRNA (Parker and Herzog, 1999) (Fig. 1), indicating that in the hypothalamus Y2 receptors may modulate the orexigenic drive of NPY, where as in the NTS, Y2 receptors may be involved is some aspects of satiety.

The Central Melanocortin System: mediator of anorexigenic drive and energy expenditure

A second family of neuropeptides, the melanocortins, also plays an important role in regulating energy homeostasis. This finding resulted from studies on monogenic obesity in mice. Five naturally occurring mutations in mice are known to cause obesity: *agouti* (A^y), *fat, tubby, obese* (Lep^{ob}/Lep^{ob}), *and diabetes* ($Lepr^{db}/Lepr^{db}$) (Friedman and Leibel, 1992). A*gouti* (A^y), the oldest mouse model of obesity described, is caused a genomic rearrangement, resulting in the ubiquitous overexpression of the agouti protein leading to the development of the agouti phenotype – consisting of yellow coat color, hyperphagia, obesity, hyperinsulinemia, and increased linear growth (Yen et al., 1994). At the level of the skin, the agouti protein inhibits alpha- melanocyte-stimulating hormone (α-MSH)-induced eumelanin production by acting to antagonize the melanocortin 1 receptor (MC1-R) (Lu et al., 1994), resulting in yellow coat color. Additionally, this secreted cysteine-knot protein was found to be an antagonist of the centrally expressed MC4-R. Inhibition of this centrally-expressed G-protein coupled receptor is now thought to be the cause of obesity in this model (Fan et al., 1997b; Huszar et al., 1997). Agouti-related peptide (AgRP), an endogenous central antagonist of the MC4-R and the MC3-R, was isolated based on homology to agouti (Ollmann et al., 1998; Shutter et al., 1997). AgRP is primarily expressed in orexigenic NPY neurons of the ARC nucleus of the hypothalamus (Broberger et al., 1998; Hahn et al., 1999) and in the adrenal gland. In the CNS, AgRP neurons project to many of the same sites as α-MSH neurons. This led to the identification of a central system capable of integrating orexigenic and anorexigenic drives in the regulation energy homeostasis– the central melanocortin system.

The central melanocortin system consists of the proopiomelanocortin (POMC) pro-hormone, which is differentially cleaved to produce the melanocortin peptides, adrenocorticotropic hormone (ACTH), α, β, and γ-MSH, and the two central melanocortin receptors MC3-R and MC4-R that modulate energy homeostasis (Cone, 1999). In addition to the endogenous melanocortin agonists and their receptors, the central melanocortin system also includes AgRP, a competitive antagonist/inverse agonist at the MC3-R and MC4-R (Ollmann et al., 1997). POMC neurons are found in only two brain areas, the ARC of the hypothalamus and the NTS of the brainstem (Bronstein et al., 1992). The ARC POMC neurons are adjacent to and receive synaptic input from AgRP/NPY neurons. The interplay between these two populations of neurons contributes to the homeostatic regulation of energy balance (Batterham et al., 2002; Cowley et al., 2001a). AgRP mRNA expression increases up to 10 fold following fasting in rodents, whereas POMC mRNA expression levels decrease (Mizuno et al., 1996). Furthermore, the reverse trends are seen with diet induced obesity (DIO) (Mizuno et al., 1996).

A number of transgenic animals with defects in the central melanocortin system offer further indication of its importance in energy balance. MC4-R (Huszar et al., 1997) and POMC knockout mice (Yaswen et al., 1999), display a characteristic melanocortin obesity phenotype, similar to the A^y phenotype described earlier, typified by hyperphagia, and metabolic defects leading to obesity. Furthermore, AgRP over-expressing mice have an identical phenotype due to the actions of the endogenous antagonist at the MC3-R and the MC4-R (Graham et al., 1997; Ollmann et al., 1997). Administration of MC3/4-R agonists (e.g. MTII), or antagonists (e.g. SHU9119), causes a transient induction or suppression of food intake respectively in wild type mice (Fan et al., 1997a) reinforcing

the data from the genetic studies. Perhaps most importantly, the melanocortin obesity phenotype is also seen in humans with POMC or MC4-R mutations, indicating that these laboratory models provide a good tool for studying the role of the central melanocortin system in regulating energy homeostasis in humans. Indeed, up to 5% of cases of monogenic obesity in adults and children are caused by defects in the MC4-R (Farooqi et al., 2003; Farooqi et al., 2000; Yeo et al., 1998).

In summary, the central melanocortin system plays a pivotal role in the regulation of energy homeostasis. Decreasing the activity of the MC4-R, by genetic or pharmacological approaches, results in an obesity syndrome. POMC and AgRP are both expressed in the ARC of the hypothalamus, project to similar sites and receive and integrate hormonal signals. Additionally, POMC neurons are also expressed in the NTS of the brainstem. Like the ARC, the NTS is adjacent to a circumventricular organ and integrates hormonal signals. However, it is also perfectly situated to receive and respond to GI signals through the vagus nerve. Furthermore, AgRP is neither expressed nor present in nerve terminals in the brainstem, a feature that distinguishes the brainstem melanocortin system from that found in the ARC. This difference implies that the brainstem POMC signaling may be differently regulated than that of the hypothalamus (Fig. 2).

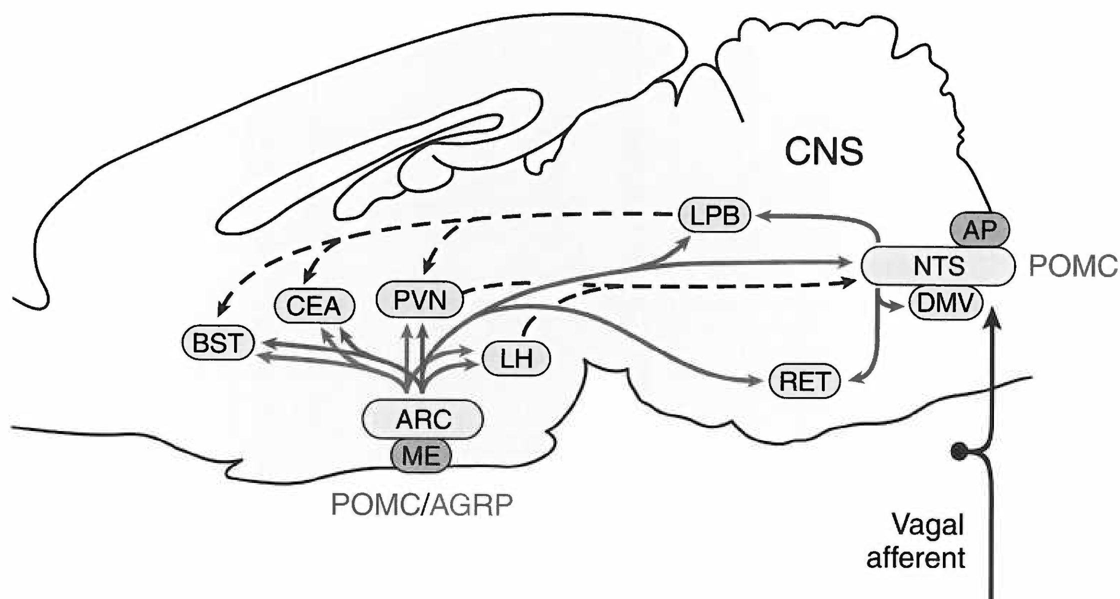

Fig. 2. Both the arcuate (ARC) nucleus of the hypothalamus and the nucleus tractus solitarius (NTS) of the brainstem express POMC neurons and receive and integrate humoral signals while the NTS additionally receives vagal afferents. Blue, nuclei containing POMC neurons; magenta, circumventricular organs adjacent to POMC neurons; yellow, a small sample of representative nuclei containing MC4R-positive neurons that may serve to integrate adipostatic and satiety signals; red arrows, representative POMC projections; blue arrows, representative AgRP projections; dashed arrows, secondary projections linking POMC neurons in hypothalamus and brainstem with common effector sites; AP, area postrema; ARC, arcuate nucleus; BST, bed nucleus of the stria terminalus; CEA, central nucleus of the amygdala; DMV, dorsal motor nucleus of the vagus; LH, lateral hypothalamic area; LPB, lateral parabrachial nucleus; ME, median eminence; NTS, nucleus tractus solitarius; PVN, paraventricular nucleus of the hypothalamus; RET, reticular nucleus of the brainstem. Figure modified from (Cone, 2005).

The ARC nucleus NPY/AgRP and POMC Circuitry Regulating Energy Balance: Transgenic, Neuroanatomical and Electrophysiological Studies

In the ARC, NPY/AgRP and POMC neuronal populations have been shown to be key mediators of energy homeostasis, integrating central and peripheral signals and relaying information about energy availability and expenditure. NPY/AgRP neurons co-localize with GABA (Horvath et al., 1997), a major inhibitory neurotransmitter in the brain, and a variety of hormone receptors, including the long form (active form) of the leptin (Lep-R) (Hakansson et al., 1998; Mercer et al., 1996b), insulin (Insulin-R) (Baskin et al., 1987) and growth hormone secretagogue receptors (GHS-R) (Willesen et al., 1999). Intriguingly, many of those same receptors, like Lep-R (Cheung et al., 1997) and Insulin-R (Benoit et al., 2002), are also found on POMC neurons in the ARC, raising an interesting question: How do two opposing neuronal populations, one orexigenic and the other anorexigenic, produce the physiologically appropriate output in response to the same peripheral signals of energy availability and storage, to maintain energy balance? Indeed, measuring mRNA levels of different neuropeptide genes, by *in situ* hybridization, has reveled that exogenous administration of leptin, an adipostatic factor released in proportion to fat, blunts the overexpression of NPY mRNA (Ahima et al., 1996; Schwartz et al., 1996; Stephens et al., 1995) and reverses the reduction in POMC mRNA expression (Mizuno et al., 1998; Schwartz et al., 1997; Thornton et al., 1997) associated with fasting or as found in Lep^{ob}/Lep^{ob} mice. Additionally, leptin induces c-Fos, a marker of neuronal activation, in POMC neurons but not in NPY/AgRP neurons (Elias et al.,

1999), presumably resulting in a net anorexigenic drive via the central melanocortin circuitry.

However, the ability to directly test ARC nucleus POMC and NPY/AgRP neuronal firing in response to a variety of hormones and nutrients was not possible until the development of an electrophysiological model system. The creation of two transgenic lines of mice; one using the brain specific POMC promoter driving expression of enhanced green-fluorescent protein (POMC-EGFP) and the other by expression of an EGFP variant, sapphire, in NPY neurons (NPY-Sapphire) was instrumental in the understanding of basic as well as inducible properties of ARC NPY/AgRP and POMC neurons. Whole cell patch clamp and loose cell recordings were used to determine the baseline firing (Takahashi and Cone, 2005) and the electrophysiological responses to a variety of hormones/ligands of NPY-Sapphire (Cowley et al., 2001b) and POMC-EGFP neurons (Batterham et al., 2002; Cowley et al., 2001b; Cowley et al., 2003). From those studies a model of the intra-ARC neuronal circuitry emerged (Fig. 3), in which ARC POMC are innervated by spontaneously firing GABA-secreting NPY neurons (Cowley et al., 2001b; Takahashi and Cone, 2005). The latter was shown by applying NPY in the bath either in the presence or absence of tetrodotoxin (TTX), blocking all action potential dependent synaptic transmission (Cowley et al., 2001b). Both in the presence and absence of TTX, NPY caused a reduction in the frequency of inhibitory post-synaptic currents (IPSCs) and mini IPSCs onto POMC cells (Cowley et al., 2001b), indicated that the inhibition of IPSCs on to POMC neurons was occurring through direct effects on NPY presynaptic nerve terminals. Indeed, both leptin and Y2 receptors, a presynaptic autoinhibitory

receptor, are expressed on NPY neurons in the ARC (Broberger et al., 1997; Hakansson et al., 1998). Furthermore, bath applied NPY also had direct effects on POMC firing, confirming the presence of an inhibitory NPY receptor, the Y1 receptor, on POMC cells (Broberger et al., 1997; Cowley et al., 2001b). This electrophysiological preparation demonstrated that MC3-R is an autoinhibitory receptor on POMC neurons and can modulate the IPSCs onto POMC neurons received by NPY neurons (Cowley et al., 2001b).

The NPY/AgRP and POMC circuit in the ARC nucleus of the hypothalamus is uniquely situated between the third ventricle and the median eminence (ME) (a circumventricular organ) to sample factors in both blood and the cerebrospinal fluid.

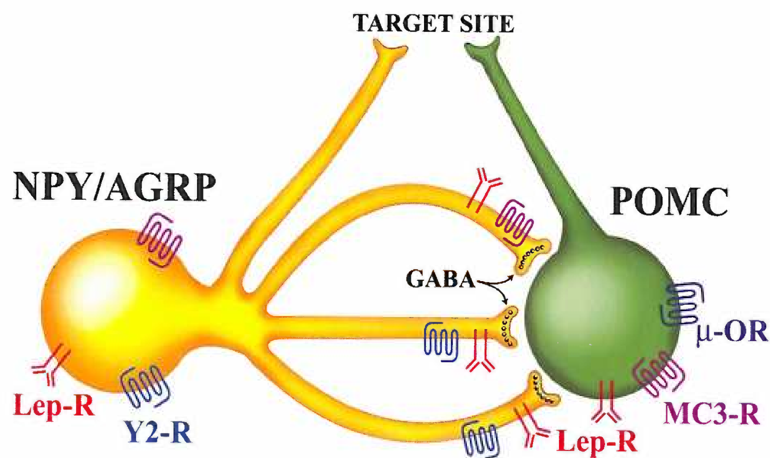

Fig. 3. Schematic drawing of the electrophysiological findings depicting the synaptic contacts and receptors involved in the arcuate NPY/AgRP and POMC circuitry. ARC NPY/AgRP neurons make inhibitory GABAergic synapses on POMC neurons. Both POMC and AgRP neurons express a variety of receptors for peripheral hormones. NPY/AgRP neurons could be autoinhibited by Y2 receptors, expressed presynaptically on NPY/AgRP neurons. MC3-R acts as inhibitory receptor on both NPY/AgRP and POMC neurons. (Cowley et al., 2001b)

Peripheral Hormones Relaying Information of Energy Expenditure and Availability Through the Central NPY/AgRP and POMC Circuitry

Long-Term Energy Reserves

Leptin

The adipostatic hormone leptin relays information about energy availability between the periphery and brain and helps to regulate energy balance. White adipose tissue produces and releases this peptide hormone (Zhang et al., 1994) in proportion to fat mass. In the late 1960's and early 1970's, Coleman and colleagues first hypothesized that a peptide like leptin might exist after studying two mutant mouse strains, the Lep^{ob}/Lep^{ob} and $Lepr^{db}/Lepr^{db}$ mice (Coleman, 1973; Coleman and Hummel, 1969), both characterized by hyperphagia, obesity, and infertility. Coleman performed parabiosis experiments, creating a cross-circulation between Lep^{ob}/Lep^{ob}, $Lepr^{db}/Lepr^{db}$ and wild type mice. He found that linking the circulation of a Lep^{ob}/Lep^{ob} and wild-type mouse led to a reduction in body weight of the obese mouse, while linking the circulatory systems of a wild-type and $Lepr^{db}/Lepr^{db}$ mice did not alter the obesity in the $Lepr^{db}/Lepr^{db}$ animal. Additionally, linking the circulatory system of a $Lepr^{db}/Lepr^{db}$ to that of a Lep^{ob}/Lep^{ob} mouse led to a reduction in body weight in the Lep^{ob}/Lep^{ob} animal. Thus, he hypothesized that the Lep^{ob}/Lep^{ob} mice were probably deficient in a circulating ligand while the $Lepr^{db}/Lepr^{db}$ mice may be lacking its receptor. It would be another twenty years, before the leptin gene was identified by positional cloning (Zhang et al., 1994) followed shortly thereafter

by the cloning of the leptin receptor (Tartaglia et al., 1995). Human genetic screens showed that mutations either in the gene encoding leptin (Montague et al., 1997; Strobel et al., 1998) or in the leptin receptor (Clement et al., 1998) result in phenotype similar to the mouse models, typified by morbid obesity, hyperphagia, and failure to undergo puberty.

The long form of the leptin receptor is principally expressed in the brain (Cheung et al., 1997; Elmquist et al., 1998; Fei et al., 1997; Guan et al., 1997b; Mercer et al., 1996b; Schwartz et al., 1996). In the hypothalamus, its expression is fairly restricted to ARC, VMH, DMH, and the ventral premammillary nuclei (VPMH) (Cheung et al., 1997; Elmquist et al., 1998; Fei et al., 1997; Guan et al., 1997b; Mercer et al., 1996b; Schwartz et al., 1996), all key areas in the regulation of energy homeostasis. Indeed, peripheral treatment of mice with gold thioglucose, causing ablation of areas involved in sensing peripheral signals and regulating energy homeostasis, markedly reduces the expression of the long form of the leptin receptor in the hypothalamus (Fei et al., 1997).

Two molecular probes have proved to be particularly useful in mapping the effects of leptin in the hypothalamus. The first, the expression of c-Fos, an immediate early gene and a marker of neuronal activation, has shown that systemic leptin administration activates neurons in the LH, retrochiasmatic area, VMH, DMN, ARC and VPMH, in close correlation with sites expressing the long form of the leptin receptor (Elmquist et al., 1997; Van Dijk et al., 1996; Woods and Stock, 1996). Another molecular marker of leptin receptor activation is the suppressor of cytokine signaling-3 (SOCS-3) (Bjorbaek et

al., 1998). The SOCS family of proteins are rapidly induced with activation of the cytokine family of receptors, of which the leptin receptor is a member (Tartaglia, 1997; White et al., 1997). In general, SOCS proteins act to inhibit the phosphorylation of STAT proteins by JAK tyrosine kinases. Peripheral administration of leptin induces SOCS-3 mRNA in neurons expressing the long form of leptin receptor (Bjorbaek et al., 1998).

There are a number of pieces of evidence that implicate the ARC NPY/AgRP and POMC circuitry in mediating some of the effect of leptin on energy homeostasis. Firstly, both NPY/AgRP and POMC neurons express Lep-R at high levels in the ARC (Broberger et al., 1997; Cheung et al., 1997; Mercer et al., 1996a). However, unlike POMC neurons, systemic administration of leptin does not induce c-Fos expression in the NPY/AgRP neurons (Mercer et al., 1996a) but instead induction of SOCS-3 mRNA is observed (Bjorbaek et al., 1998), indicating that they are not activated by leptin but may instead be inhibited, suggesting a differential effect of the peptide in orexigenic and anorexigenic neurons. Additionally, crossing Lep^{ob}/Lep^{ob} with $NPY^{-/-}$ mice, as described previously, produces a partial reversal of the Lep^{ob}/Lep^{ob} phenotype (Erickson et al., 1996a) suggesting that compensatory increases in NPY may contribute to the obesity seen in the Lep^{ob}/Lep^{ob} mouse. In contrast to its effects on ARC NPY/AgRP neurons, leptin induces c-Fos and SOCS-3 mRNA in POMC neurons (Elias et al., 1999), indicating the activation of ARC POMC neurons following peripheral leptin administration. Furthermore, the decrease of POMC mRNA after fasting or in Lep^{ob}/Lep^{ob} mice can be prevented by peripheral leptin administration (Mizuno et al., 1998; Schwartz et al., 1997; Thornton et

al., 1997). Finally, central administration of melanocortin receptor antagonist can reverse the reduction in food intake and the activation of the sympathetic nervous system caused by leptin administration (Satoh et al., 1998; Seeley et al., 1997).

The direct effects of leptin and other factors on the electrophysiological properties of POMC and NPY/AgRP neurons has been examined using POMC-EGFP (Cowley et al., 2001b) and NPY-Sapphire mice (Roseberry et al., 2004). Whole cell patch clamp recordings showed that bath-applied leptin increased the firing rate of POMC neurons (Cowley et al., 2001b). This effect was found to be both direct and indirect; leptin activated a non-specific cation channel on POMC neurons, and inhibited GABA release from NPY neuron terminals terminating on POMC neurons (Cowley et al., 2001b). The downstream effects of leptin are mediated in part in the PVN and LH, two nuclei known to receive projections from the ARC NPY/AgRP and POMC neuronal populations. In those nuclei, the relative reduction of the tonic orexigenic drive by NPY/AgRP neurons and the concomitant increase of anorexigenic drive from POMC neurons. This produces weight loss by simultaneously reducing food intake and increasing energy expenditure.

Regulation of Energy Intake via Acute Satiety and Hunger Pathways

Leptin communicates information to the CNS regarding levels of long-term energy stores. Not surprisingly in this regard, leptin has been found to regulate circuits involved in acute control of energy intake and expenditure. Hunger and satiety are regulated by both neural and humoral inputs to the ARC and NTS. The NTS is a major site in the

brainstem capable, like the ARC, of sampling the circulation due to its close proximity to a circumventricular organ, the area postrema (AP), but also receives information from the periphery directly via the vagus nerve. Furthermore, the NTS is the only other CNS site, besides ARC, to express both POMC and NPY. However, the brainstem does not express AgRP, the endogenous melanocortin receptor antagonist. This difference implies that the brainstem POMC signaling may be differently regulated than that of the hypothalamus. Future studies are necessary to elucidate the regulation of brainstem POMC expression, cleavage, and signaling.

Ghrelin

In 1977 and later in 1980, Bowers reported that a synthetic peptide analogue of met-enkephalin induces the release of growth hormone (GH) in vitro, and it was accordingly named GH secretagogue (Bowers et al., 1980). Since then, numerous GH secretagogue peptides have been described, including growth hormone releasing peptide-1 (GHRP)-1, GHRP-2, GHRP-6, hexarelin, and ipamorelin (for review see (Smith et al., 2001)). Ghrelin is the most recently characterized endogenous growth hormone secretagogue (Kojima et al., 1999). The highest level of ghrelin expression is found in the fundus of the stomach (Kojima et al., 1999) but the pancreas (Date et al., 2002; Volante et al., 2002a), the immune cells (Hattori et al., 2001), the lung (Volante et al., 2002b), the testis (Barreiro et al., 2002), the ovary (Caminos et al., 2003), the placenta (Gualillo et al., 2001), the kidney (Mori et al., 2000), the pituitary (Korbonits et al., 2001), and the brain (Cowley et al., 2003; Lu et al., 2002) have also been shown to express ghrelin, albeit at

lower levels. Even though ghrelin is classified as a GH secretagogue, it does not act via the known GHRP receptor, instead it mediates its actions through a separate growth hormone secretagogue receptor (GHS-R) (Howard et al., 1996; McKee et al., 1997). The hypothalamus has the highest levels of GHS-R expression (Bennett et al., 1997; Guan et al., 1997a) followed by the pituitary (Howard et al., 1996; Korbonits et al., 1998). In particular, the ARC, PVN, suprachiasmatic nucleus, anterolateral hypothalamic nucleus, anteroventral preoptic nucleus, and tuberomamaillary nucleus of the hypothalamus express GHS-R message (Bennett et al., 1997; Guan et al., 1997a). Peripheral expression of GHS-R is controversial, but using the sensitive real-time PCR technique, GHS-R mRNA has been found in human adrenal, thyroid, pancreas, myocardium, spleen, and testes (Gaytan et al., 2004). Peripheral ghrelin secretion is tightly linked to the nutritional status of an organism. Fasting causes elevation of serum ghrelin levels whereas food consumption causes a rapid fall in circulating ghrelin (Ariyasu et al., 2001; Cummings et al., 2001; Cunha and Mayo, 2002). In contrast, feeding has no effect on pituitary or hypothalamic ghrelin expression (Torsello et al., 2000). The mechanism by which ghrelin levels fall after food intake is an important area of investigation; it has been postulated that the meal related ghrelin decrease requires a postgastric factor. This phenomenon has been illustrated by experiments where gastric distention is controlled via pyloric cuff, ether blocking or allowing gastric emptying, and either saline or glucose is infused into the stomach (Williams et al., 2003). Neither infusion of saline or glucose caused a reduction of ghrelin with the pyloric cuff inflated, blocking gastric emptying, while only glucose caused a decrease in ghrelin levels when the stomach was allowed to drain properly (Williams et al., 2003).

Ghrelin has been shown to have effects on multiple systems, including GH release, feeding, gastric acid secretion, gastric motility, adrenocorticotropic hormone (ACTH) release, and cell proliferation (for review see (Smith et al., 2001)). The first effects of ghrelin on feeding and weight gain were shown in 2000, when subcutaneous (sc) ghrelin treatment was found to cause weight gain in GH-deficient and wild-type control rats (Tschop et al., 2000). The orexigenic nature of peripherally administered ghrelin has been repeated in multiple species (Asakawa et al., 2003; Lall et al., 2001; Nakazato et al., 2001). Repeated administration of GHS-R antagonists also decreases body weight gain and improves glycemic index in Lep^{ob}/Lep^{ob} mice (Asakawa et al., 2003). The preprandial increase in ghrelin levels correlates well with initiation of meals and hunger scores in humans, suggesting a possible role of the peptide in meal initiation (Cummings et al., 2004).

The effects of ghrelin on food intake are thought to be predominantly mediated centrally by the mediobasal/mediolateral hypothalamus and the brainstem. Central administration of ghrelin into the third ventricle, fourth ventricle or directly into the ARC or PVN increases food intake, and induces the expression of c-Fos in the ARC, PVN, DMH, and LH of the hypothalamus and the NTS and AP of the brainstem (Bagnasco et al., 2003; Date et al., 2001; Faulconbridge et al., 2003; Lawrence et al., 2002; Olszewski et al., 2003a; Torsello et al., 2000; Wren et al., 2001; Wren et al., 2000). However, a very different pattern c-Fos neuronal activation is observed after peripheral administration of ghrelin, with the main central site of c-Fos expression restricted to the ARC of the

hypothalamus (Dickson et al., 1995; Dickson et al., 1993; Hewson and Dickson, 2000). Within the ARC, acute and chronic peripheral administration of ghrelin causes an increase in both AgRP and NPY mRNA (Asakawa et al., 2001; Kamegai et al., 2001). Indeed, the most ventral ARC, which lies next to the ME expresses high levels of GHS-R on NPY/AgRP neurons (Willesen et al., 1999). Furthermore, these neurons form extensive axo-somatic and axo-dendritic contacts with central neurons expressing ghrelin, which are found in the internuclear space between the LH, ARC, VMH, DMH and PVH (Cowley et al., 2003; Lu et al., 2002).

From these observations, it was postulated that centrally ghrelin may have the opposite effects on the ARC nucleus melanocortin circuitry compared to leptin. Indeed, ghrelin has subsequently been shown to activate NPY/AgRP neurons (Asakawa et al., 2001; Cowley et al., 2003; Kamegai et al., 2001), which are known to be inhibited by leptin, and to inhibit POMC neurons (Cowley et al., 2003), activated by leptin. In an electrophysiological slice preparation, bath applied ghrelin acted directly on ARC NPY/AgRP neurons to increase their firing, resulting in the increase of GABAergic IPSCs onto POMC neurons thereby causing their inhibition (Cowley et al., 2003; Riediger et al., 2003) (Fig.4). Further evidence that the central melanocortin system plays a crucial role in mediating the orexigenic actions of ghrelin (Chen et al., 2004) comes from A^y; (Martin et al., 2004)), $NPY^{-/-}$, MC3-R/MC4-R and NPY/AgRP double knockout mice, which all show an attenuated feeding response following peripheral ghrelin administration, compared to wild-type animals. Finally, the importance of the ARC in mediating the orexigenic actions of ghrelin is further emphasized by experiments

in mice with monosodium glutamate (MSG) induced ARC lesions. MSG treatment abolishes the orexigenic feeding response to peripherally administered ghrelin (Tamura et al., 2002). In addition to the central melanocortin system, orexin has also been implicated in mediating some of the actions of ghrelin on food intake (Lawrence et al., 2002; Olszewski et al., 2003b; Toshinai et al., 2003).

The ARC NPY/AgRP neurons are thought to mediate the orexigenic effects of peripheral and central ghrelin, but it is important to note that numerous gut-released peptides utilize the vagus and the DVC to relay and/or mediate their actions. GHS-R are expressed on the vagus (Date et al., 2002; Sakata et al., 2003) and in the brainstem (Katayama et al., 2000). Indeed, like CCK, the effects of ghrelin on food intake require an intact vagus nerve (Asakawa et al., 2001; Date et al., 2002) and peripheral administration of ghrelin induces c-Fos in numerous brainstem sites known to receive projections from the NTS and the vagus, such as the PVH (Ruter et al., 2003) and inferior olivary nucleus (Zhang et al., 2003). However, there is a notable absence of c-Fos expression in the DVC, an area known to receive inputs from the vagus, after peripheral administration of ghrelin (Ruter et al., 2003; Zhang et al., 2003). One possible explanation for this finding is that ghrelin may inhibit neurons in the DVC, a satiety center, and therefore not cause c-Fos expression in that region. Furthermore, in their study, Date et al., showed that c-Fos expression in the ARC nucleus is abolished following vagotomy (Date et al., 2002) indicating that connectivity to the vagus nerve, via centers relaying its effects such as the DVC, may be essential for c-Fos activation in the ARC and subsequent orexigenic response to ghrelin. Finally, site-specific injections of ghrelin into the DVC produce

profound hyperphagia, suggesting that the DVC, like the ARC, may be involved in relaying its orexigenic effects.

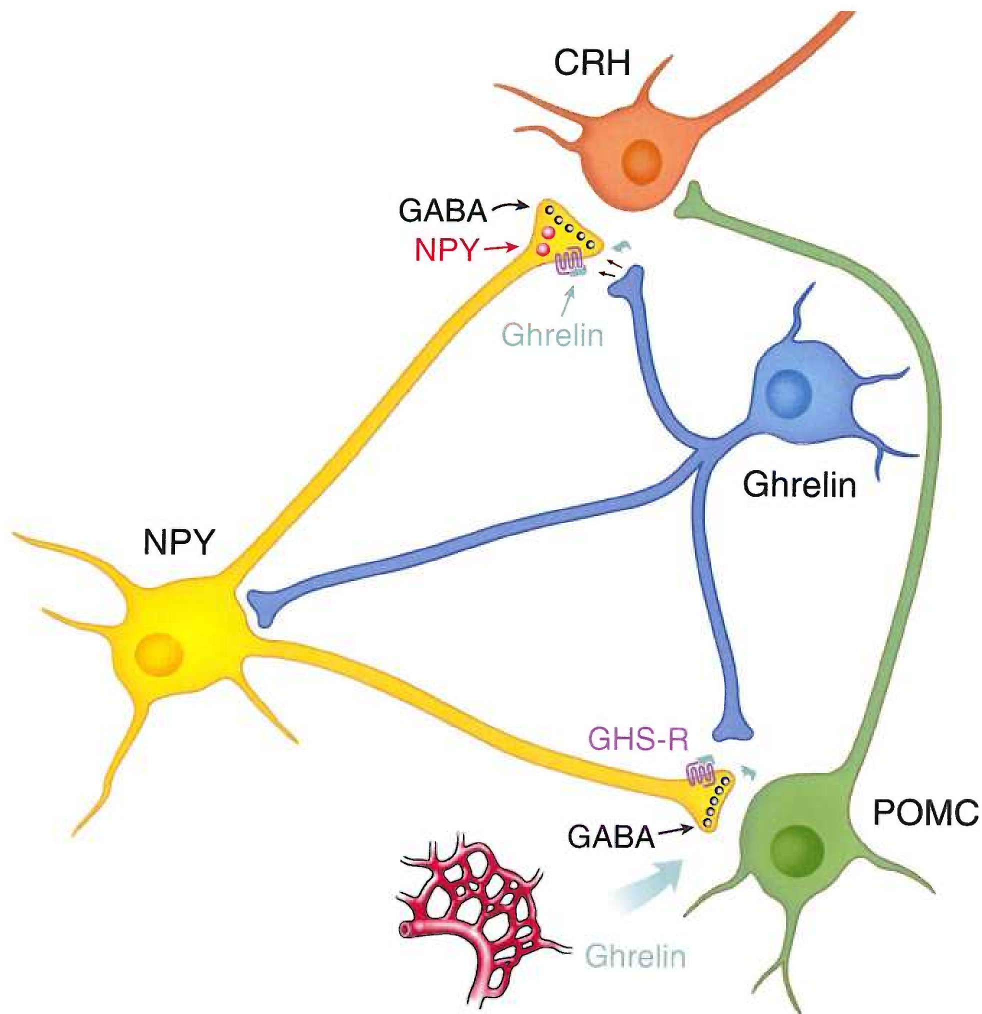

Fig. 4. Ghrelin, originating either from the vasculature or from central ghrelin neurons, binds presynaptic GHS-R receptors on arcuate NPY/AgRP neurons to induce GABA release and inhibit POMC neurons. In the paraventricular nucleus, central ghrelin activates NPY/AgRP presynaptic terminals to inhibit CRH neurons. (Cowley et al., 2003)

Cholecystokinin (CCK)

Cholecystokinin (CCK), first identified over 30 years ago, has become the archetypal gut-released satiety factor. CCK is released from the proximal small intestine in response to ingested foods. In particular, dietary fat and protein are most potent at inducing CCK release, locally and into the circulation (Liddle et al., 1985). The levels of CCK gradually increase, beginning at 10 min after meal initiation, reaching a peak in the circulation at 30 min, and remaining elevated for up to 5 h after eating (Liddle et al., 1985).

One of the key actions of CCK at the level of the GI tract was found to be the maximization of nutrient absorption. CCK, binding to CCK-A receptors inhibits gastric emptying, induces pancreatic enzyme secretion and causes gallbladder smooth muscle contractions, releasing bile in the duodenum (Liddle et al., 1985; Moran et al., 2001; Moran and McHugh, 1982; Owyang, 1996).

In an early report, Smith et al., identified CCK as a potential humoral factor inhibiting food intake and proposed a framework by which a peptide or hormone qualifies as a satiety factor (Smith et al., 1981). In short, the following criteria were proposed: 1) the factor/hormone has to be activated in response to feeding, 2) exogenous administration of the factor/hormone should cause a dose dependent inhibition of feeding, 3) the factor/hormone's anorexigenic effect should be of rapid onset and short-lived to respond to ingestive behavior, 4) the anorexigenic effect of the factor/hormone should not be due

to illness, like aversion, and 5) the factor/hormone should be effective at physiological (post-meal) concentrations to inhibit feeding. Many of these criteria are still used and applied to a growing number of newly discovered peptides regulating satiety.

Exogenous administration of CCK reduces food intake in the short-term in a number of species including rodents (Gibbs et al., 1973) and humans (Della-Fera and Baile, 1980; Gibbs et al., 1976; Houpt et al., 1978; Pi-Sunyer et al., 1982). Although, chronic intraperitoneal (ip) administration of CCK continues to reduce meal-size, there is a compensatory increase in meal number leading to no overall change in daily caloric intake or body weight (West et al., 1984).

Although, the satiating effects of CCK have been studied for over 30 years, a molecular mediator for its anorexigenic actions has proven to be elusive until recently. Early studies showed that the vagus nerve is essential for the satiating effects of CCK (Ritter and Ladenheim, 1985; Smith et al., 1985). Indeed, CCK activates CCK-A receptors on vagal afferents (Ritter and Ladenheim, 1985; Smith et al., 1985), to relay satiety information to the NTS of the brainstem. However, only recently, the MC4-R in the brainstem have been implicated in mediating the anorexigenic effects of CCK (Fan et al., 2004). The first link between CCK and central melanocortin system was suggested by studies in MC4-R knockout mice (Butler et al., 2001). In addition to the well-characterized melanocortin obesity phenotype, MC4-R knockout mice also have a defect in their ability to respond appropriately to changes in fat in the diet (Butler et al., 2001). When placed on a moderate fat diet, wild type mice will reduce their food intake to

compensate for the increase in caloric value of the food, whereas MC4-R knockout mice further increase their food intake, suggesting an inability to respond appropriately to this change (Butler et al., 2001). Additionally, numerous feeding preference studies have also implicated the melanocortin system in the control of fat consumption (Hagan et al., 2001; Koegler et al., 1999; Samama et al., 2003). Furthermore, like a satiety factor, central administration of the melanocortin agonist, MTII, causes a reduction of meal-size but not frequency (Azzara et al., 2002; Williams et al., 2002). These observations combined with the fact that CCK is released in response to fatty acids in the gut, suggested that MC4-R knockout mice might have a defect in the response to CCK, contributing to the obesity seen in MC4-R knockouts. Indeed, MC4-R knockout mice have a largely attenuated response to the anorexigenic effects of CCK (Fan et al., 2004), suggesting that the MC4-R is important for the actions of CCK on food intake. Additionally, peripheral administration of CCK causes activation of ~30% of NTS POMC neurons in POMC-EGFP mice, and sub-threshold 4th ventricle administration of SHU9119, a non-specific MC3/4-R antagonist, reversed its satiating effects (Fan et al., 2004). Additional evidence that NTS POMC neurons may mediate the satiating effects of CCK comes from recent report by Appleyard et al in 2005, which showed that, in an electrophysiological slice preparation, NTS POMC neurons receive direct connections from the solitary tract (Appleyard et al., 2005). Furthermore, bath application of CCK increased the amplitude of the solitary tract-stimulated excitatory post-synaptic currents (EPSCs) and frequency of miniature EPSCs recorded from NTS POMC neurons (Appleyard et al., 2005). Thus, the central melanocortin system appears to play a role in the regulation of energy

homeostasis in the long-term, via ARC POMC neurons that respond to leptin, and in the short-term via NTS POMC neurons, mediating the actions of CCK.

As stipulated by the Smith criteria (Smith et al., 1981), a satiety factor has to produce short-term inhibition of food intake without causing aversion. Indeed, at physiological concentrations, CCK acts as a satiety factor (Smith et al., 1981) however, at high concentrations, peripheral CCK causes vagally mediated taste aversion (Ervin et al., 1995; Verbalis et al., 1986). The aversive actions of CCK are thought to involve the activation of AP neurons (Luckman, 1992), which have been implicated in mediating the response to aversive stimuli (Miller and Leslie, 1994). In contrast to other aversive agents, like LiCl, the aversive effects of CCK can be reversed by anti-emetic drugs (McCann et al., 1989) or vagotomy (Martin et al., 1978; Verbalis et al., 1986), indicating that they work through different pathways. In the literature there is often a clear distinction made between satiety and aversion. However, unlike toxins, which reduce or halt food intake through purely aversive mechanisms, CCK reduces food intake via homeostatic mechanisms, thus in this case aversion may just be an extreme form of satiety (Verbalis et al., 1986). We have all eaten one too many slices of pizza to the point of nausea but that nausea is very different than the nausea induced by food poisoning.

CCK has been shown, both centrally and peripherally, to have a synergistic interaction with leptin (Barrachina et al., 1997) in regulating food intake. Changes in circulating leptin either due to exogenous administration (Barrachina et al., 1997), the estrus cycle, or fasting-induced reduction in adipose mass (Maffei et al., 1995) affect the efficacy of

CCK to reduce food intake. Likewise, rodents with defective leptin signaling, such as obese Zucker rats (McLaughlin and Baile, 1980; Niederau et al., 1997), show desensitized response to bolus injections of CCK. A central mechanism for these observations was proposed by Emond and colleagues where leptin might enhance the effects of CCK by altering the excitability of neurons in the NTS through the activation of descending pathways from the hypothalamus (Emond et al., 1999a). Also, peripheral electrophysiological studies have shown that pretreatment with CCK can sensitize CCK-responsive vagal afferents (Wang et al., 1997) to leptin. In an isolated intact gastric-vagal afferent unit preparation two-types of leptin-sensitive terminals were found. The first terminal type, responsive to leptin but unaffected by pretreatment with CCK, are thought to be involved in monitoring circulating leptin and in the regulation of long-term energy homeostasis. The second terminal type, insensitive to leptin unless pretreated with CCK, are proposed to play a role in mediating satiety (Wang et al., 1997). Finally, leptin alone and in combination with CCK, has been shown to modulate calcium levels of cultured vagal afferent neurons (Peters et al., 2004), providing further evidence for peripheral interaction between leptin and CCK at the level of the vagus.

Peptide Tyrosine Tyrosine (PYY)

PYY is the third member of the PP family, which includes neuropeptide tyrosine (NPY) and PP. PYY has two major endogenous forms, PYY_{1-36} and PYY_{3-36}. Nutrient content of a meal causes the direct or indirect release of PYY_{1-36}, post-prandially, into the blood stream (Ballantyne et al., 1989; Cherbut et al., 1998; Dumoulin et al., 1998; Fu-Cheng et

al., 1997; Gomez et al., 1996; Lin et al., 2000; Longo et al., 1991; Plaisancie et al., 1996; Sheikh et al., 1989; Taylor, 1985; Zhang et al., 1993a). In the circulation, approximately 40% of PYY_{1-36} is cleaved by dipeptidyl peptidase-IV (DPPIV) to produce PYY_{3-36} (Grandt et al., 1994a). The two forms produced by this cleavage have different specificity for the NPY receptors. PYY_{1-36} binds to Y1, Y2, and Y5 receptors (Holliday et al., 2000; Keire et al., 2000) while PYY_{3-36} has very high affinity for the Y2 autoreceptor, with some specificity for the Y5 receptor at high concentrations (Keire et al., 2002; Keire et al., 2000).

Multiple factors govern the release of PYY_{1-36} from the gut, from intraluminal to neuro-humoral signals. Plasma PYY_{1-36} levels start raising shortly after the ingestion of a meal, reaching peak concentrations 60-90 min following meal initiation (Taylor, 1985). L-endocrine cells, found at high density at the level of the ileum and colon, with only a few scattered cells in the proximal bowel (Bottcher et al., 1993; McDonald et al., 1993), release PYY_{1-36}. L-cells release PYY_{1-36} in response to three luminal factors – chemical, osmolar, and caloric content. Fats in a meal, in particular long-chain fatty acids, are the most potent luminal factors causing the release of PYY_{1-36} (Ballantyne et al., 1989; Sheikh et al., 1989; Taylor, 1985). In addition to fatty acids, other nutrients, such as protein, amino acids (Plaisancie et al., 1996), and carbohydrate (Dumoulin et al., 1998), can also induce PYY_{1-36} release from some regions of the intestine. However, plasma PYY_{1-36} levels increase immediately after meal ingestion (Hill et al., 1991; Taylor, 1985), even before nutrients have reached the distal small intestine, suggesting an additional hormonal or neural mechanism may be involved in the release of PYY_{1-36}. Indeed,

humoral signals, like gastric acid (Gomez et al., 1996) and CCK (Lin et al., 2000), can potently induce the secretion of PYY_{1-36} from the ileum, independent of intraluminal contents. Furthermore, cholinergic signals, both muscarinic and nicotinic, originating from the proximal small intestine, have been shown to cause PYY_{1-36} release from L-cells (Fu-Cheng et al., 1995; Fujimiya et al., 1992), presumably involving the vagus nerve. These observations have reveled a very complex system of direct and indirect regulation of PYY_{1-36} release.

In the GI tract, PYY_{1-36} functions as the "ileal and colonic brake", inhibiting proximal intestinal secretions and motility, to allow the absorption of calorie dense nutrients (Al-Saffar et al., 1985; Ballantyne et al., 1989; Cherbut et al., 1998; Fox-Threlkeld et al., 1993; Krantis and Harding, 1991; Wager-Page et al., 1993a; Wager-Page et al., 1993b; Wager-Page et al., 1993c). PYY_{1-36}, in an *in vitro* preparation, induces the contraction of circular ileal and colonic muscles (Ballantyne et al., 1989; Fox-Threlkeld et al., 1993) while relaxing the longitudinal muscles of the duodenum, jejunum, ileum, and colon (Krantis and Harding, 1991) to produce a net reduction of peristalsis in the GI tract. Furthermore, in an *in vivo* preparation in anesthetized rats, PYY_{1-36}, as well PYY_{3-36}, increases duodenal and colonic intramural pressure, retarding the transit time of intestinal contents (Al-Saffar et al., 1985; Cherbut et al., 1998; Wager-Page et al., 1993a; Wager-Page et al., 1993b; Wager-Page et al., 1993c). However, an interesting paradoxical phenomenon has been described where PYY_{1-36} reduces colonic peristalsis in an *in vitro* preparation (Al-Saffar et al., 1985; Ballantyne et al., 1989; Cherbut et al., 1998; Fox-Threlkeld et al., 1993; Krantis and Harding, 1991), while, in an in vivo preparation, it

causes an increase in colonic transit rate, reducing colonic transit time {Wager-Page, 1993 #6737; Wager-Page et al., 1993a; Wager-Page et al., 1993c). This contradiction could reflect the fact that the vagus nerve, severed in an *in vitro* preparation, may mediate some of the effects of PYY_{1-36}. Indeed, the increase in colonic transit rate is dependent on cholinergic signals coming from the vagus nerve (Wager-Page et al., 1993a; Wager-Page et al., 1993b; Wager-Page et al., 1993c). Furthermore, Pappas et al. showed that intravenous (iv) PYY_{1-36}, at levels observed after a meal, can almost completely abolish the cephalic phase of acid secretion, thought to be mediated by efferent output from the vagus nerve, while it has almost no effect on acid secretion induced by peripherally acting secretagogues, such as histamine and pentagastrin (Pappas et al., 1986). This observation was later supported by the observation that PYY_{1-36} blocked much of the effects of baclofen, a GABA-B receptor agonist known to stimulate gastric acid output, which can also be completely reversed by atropine sulfate, an anti-cholinergic drug, or vagotomy (Hashimoto et al., 1989). Finally, highly selective vagotomy (HSV) in dogs increased the necessary IC50 dose of iv PYY_{1-36} to inhibit gastric acid secretion by 4-fold (Lloyd et al., 1997). These studies raise the possibility that some of the peripheral effects of PYY_{1-36} on gut motility and gastric acid secretions are centrally mediated.

Vagal efferents arise from the DVC of the brainstem, a center pivotal for the integration, relay, and mediation of digestive function, satiety, and hunger. The NTS, AP, and dorsal motor nucleus of the vagus (DMX), which send efferent vagal projections, constitute the DVC. Initially, ip administration of PYY_{1-36} was shown to activate c-Fos, a maker of neuronal activation, in the AP and NTS (Bonaz et al., 1993), indicating that peripherally

secreted PYY_{1-36}, and the later cleavage product PYY_{3-36}, may have direct effects in the DVC. Indeed, specific binding sites of radio-labeled PYY_{1-36} and PYY_{3-36} have been shown in the AP and NTS (Dumont et al., 1996), which express Y1 and Y2 receptors mRNA at very high density (Parker and Herzog, 1999) (Fig. 1). Furthermore, microinjections of PYY_{1-36} and PYY_{13-36}, a specific Y2 receptor agonist, in the DVC at femtomolar concentrations inhibited gastric motility induced by centrally applied thyrotropin-releasing hormone (TRH) (Chen et al., 1997), gastric acid secretion and a motility inducer. Additionally, iv infusion of PYY_{1-36} inhibits intracisternal (ic) TRH analog induced gastric acid secretion (Yang et al., 2000). Strikingly, intracisternal (ic) injection of PYY antibody almost completely abolished gastric acid inhibition produced by iv PYY_{1-36} (Yang et al., 2000). These observations indicate that peripherally secreted PYY_{1-36} and PYY_{3-36} may be acting centrally to inhibit gastric acid secretion and gastric motility through the DVC and vagal efferents.

In addition to regulating GI motility and secretions, PYY_{1-36} and PYY_{3-36}, have short-term effects on food intake. As described above, like NPY, centrally administered PYY_{1-36} is one of the most potent orexigenic peptides known (Clark et al., 1984; Morley et al., 1985; Stanley and Leibowitz, 1984). The orexigenic effects of centrally administered PYY_{1-36} are mediated by the Y1 and Y5 receptors (Inui, 1999; Mullins et al., 2001; Parker et al., 2000). Although PVN administration of Y2 receptor specific agonists reduces food intake (Leibowitz and Alexander, 1991), PYY_{3-36}, which has high affinity for the Y2 receptor, when centrally administered, increases food intake in rodents (Marsh et al., 1999b; Morley et al., 1985). These effects are likely to result from the activation of the Y5 receptors by PYY_{3-36}, due to supraphysiological concentrations achieved by direct

central administration. In contrast to the highly potent central orexigenic effects of PYY_{1-36}, peripheral administration causes a reduction in food intake in rodents (Riediger et al., 2004). Indeed, it has been proposed that serum elevation of systemic PYY_{1-36} due to gastric bypass surgery or certain kinds of gastrointestinal tract inflammatory disease and cancers may cause the reduction in food intake and subsequent anorexia associated with these disorders (Batterham et al., 2002; Naslund et al., 1997).

Parallel to the effects of systemic PYY_{1-36}, peripheral administration of PYY_{3-36} has anorexigenic effects (Batterham et al., 2002). Batterham and co-workers in 2002 showed that ip administration of PYY_{3-36} reduces food intake in nocturnal and fast-induced re-feeding models in rodents (Batterham et al., 2002). Twice daily ip injections of PYY_{3-36} reduced cumulative food intake and the rate of body weight gain in rats (Batterham et al., 2002). Additional observations led to the hypothesis that the anorexigenic effects of PYY_{3-36} were mediated by the ARC NPY/POMC circuitry (Batterham et al., 2002). First, peripheral administration of PYY_{3-36} increased c-Fos immunoreactivity in a small percentage of POMC neurons in the ARC (Batterham et al., 2002), an indication that these neurons are directly or indirectly activated by PYY_{3-36}. Intra-ARC injection of PYY_{3-36} at nanomolar concentrations caused a dose-dependent reduction of food intake in rats (Batterham et al., 2002). In an ARC slice preparation, bath applied PYY_{3-36} presumably by binding Y2 receptors on NPY neurons, decreased the inhibitory inputs of NPY neurons onto ARC POMC neurons, increasing their basal firing rate (Batterham et al., 2002) (Fig. 5). Finally, in a hypothalamic explant experiment, a Y2 receptor specific agonist increased the α-MSH and decreased NPY release from nerve terminals

(Batterham et al., 2002).

From these observations, a model for the anorexigenic actions of PYY_{3-36} was proposed: PYY_{1-36}, released postprandially, is converted in the circulation to produce PYY_{3-36}. After cleavage, circulating PYY_{3-36} may gain access to the ARC through the median eminence or via blood brain barrier transport (Nonaka et al., 2003). In the ARC, PYY_{3-36} acts through Y2 receptors to inhibit NPY neurons, reducing NPY and GABA release, indirectly activating POMC neurons, and increasing α-MSH release. The combination of reduced NPY release and increased α-MSH release produces a net anorexigenic drive on downstream targets leading to a reduction in food intake (Summarized in Fig. 5).

Intriguingly, this peptide has achieved additional attention due to the observation that continuous iv infusion of PYY_{3-36} in a small number of lean and obese human subjects reduced their 24 h food intake and hunger scores, suggesting a potential clinical utility of the peptide in the treatment of obesity (Batterham et al., 2003; Batterham et al., 2002).

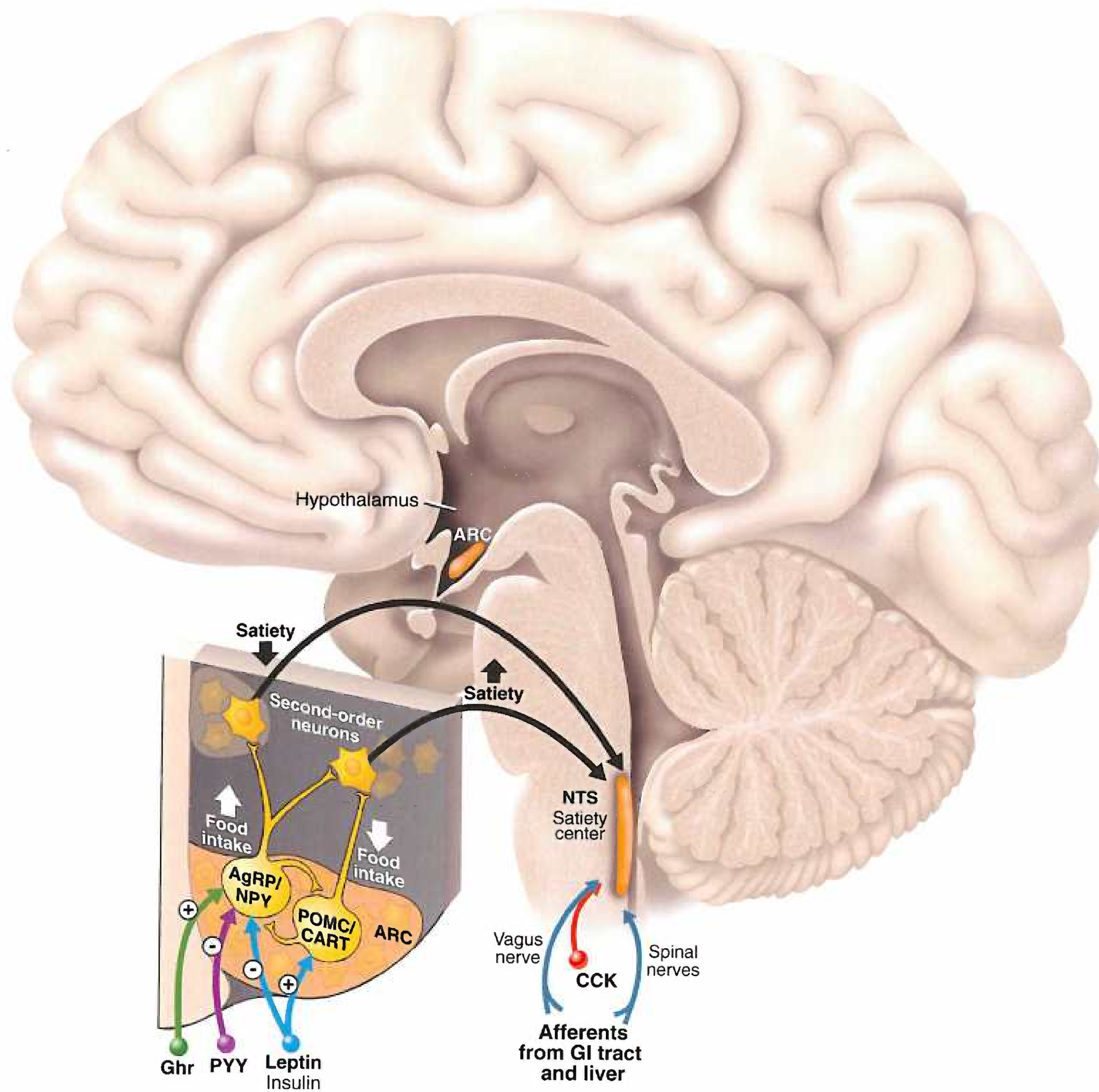

Fig. 5. Central control of energy homeostasis. The arcuate nucleus (ARC) of the hypothalamus contains the orexigenic NPY/AgRP and anorexigenic POMC neurons. The activation of NPY/AgRP neurons by low energy states, like ghrelin and fasting, increases appetite and decreases energy expenditure, where as activation of POMC neurons in an excess energy state, like high leptin levels and $PYY_{3\text{–}36}$, has the opposite effects. These neurons relay humoral information to second order neurons expressing MC4-R in nuclei such as the paraventricular nucleus and lateral hypothalamus. A second site expressing POMC neurons, receiving visceral information through the circulation and the vagal afferents, is the NTS, which mediates the effects of satiety hormones, like CCK. (Marx, 2003)

SPECIFIC AIMS AND OUTLINE OF THE THESIS

PYY_{3-36} was first demonstrated by Batterham et al. in 2002 to reduce food intake in rodents and humans (Batterham et al., 2002). Furthermore, the ARC central melanocortin system was proposed to mediate the anorexigenic actions of PYY_{3-36}. This hypothesis stemmed from the observation that peripheral administration of PYY_{3-36} caused induction of c-Fos in a small number of ARC POMC neurons (Batterham et al., 2002). In addition, in an ARC slice preparation bath applied PYY_{3-36} inhibited NPY and activated POMC neuronal firing, which was proposed to produce a net anorexigenic drive from the ARC (Batterham et al., 2002). However, previous reports and certain aspects of the original paper prompted us to further examine the proposed mechanism of action of PYY_{3-36} (Batterham et al., 2002). First, the vagus nerve and brainstem has been shown to be sufficient in mediating the satiating effects of the postprandially released gut peptide CCK, therefore, it seemed unusual that another gut peptide like PYY_{3-36} would act directly in the ARC to reduce food intake in the short-term. Indeed, as described in the introduction, PYY_{1-36} has been shown to have direct effects in the NTS of the brainstem. Additionally, ip injection of PYY_{3-36} only increased the expression of c-Fos, an indirect marker of neuronal activation, in approximately 12% of ARC POMC neurons (Batterham et al., 2002), suggesting that the role of the melanocortin system in the action of PYY_{3-36} needed to be examined more carefully. Finally, understanding the physiology of PYY_{3-36} has been further complicated by the fact that 12 collaborating laboratories have had difficulty demonstrating inhibition of feeding by the peptide in rodents (Tschop et al., 2004).

The goals of this thesis research were to:

1) Design a reliable and reproducible feeding protocol to assay the anorexigenic actions of PYY_{3-36} (examined in Chapter 1).

2) Determine to what extent the central melanocortin system mediates the anorexigenic actions of the PYY_{3-36} (examined in Chapter 1 and 3).

3) Examine if an intact ARC nucleus is required for the anorexigenic actions of PYY_{3-36} (examined in Chapter 3).

4) Examine whether other sites known to mediate the satiety effects of CCK, like the vagus nerve and the brainstem, may also be involved in mediating the anorexigenic actions of PYY_{3-36} (examined in Chapter 2 and 3).

5) Examine if peripheral administration of PYY_{3-36}, at doses that reliably and reproducibly reduce food intake, causes aversion in mice (examined in Chapter 2).

CHAPTER ONE

Peptide YY_{3-36} Inhibits Food Intake in Mice through a Melanocortin-4 Receptor-Independent Mechanism

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ABSTRACT

Peptide YY_{3-36} (PYY_{3-36}), a peptide released postprandially by the gut, has been demonstrated to inhibit food intake. Little is known about the mechanism by which PYY_{3-36} inhibits food intake, although the peptide has been shown to increase hypothalamic proopiomelanocortin (POMC) mRNA *in vivo* and to activate POMC neurons in an electrophysiological slice preparation. Understanding the physiology of PYY_{3-36} is further complicated by the fact that some laboratories have had difficulty demonstrating inhibition of feeding by the peptide in rodents. We demonstrate here that, like cholecystokinin, PYY_{3-36} dose-dependently inhibits food intake by approximately 20–45% over a 3- to 4-h period post ip administration, with no effect on 12-h food intake. This short-lived satiety effect is not seen in animals that are not thoroughly acclimated to handling and ip injection, thus potentially explaining the difficulty in reproducing the effect. Surprisingly, PYY_{3-36} was equally efficacious in inducing satiety in wild-type and melanocortin-4 receptor (MC4-R)-deficient mice and thus does not appear to be dependent on MC4-R signaling. The expression of c-Fos, an indirect marker of neuronal activation, was also examined in forebrain and brainstem neurons after ip treatment with a dose of PYY_{3-36} shown to induce satiety. The peptide induced no significant neuronal activation in the brainstem by this assay, and only modest activation of hypothalamic POMC neurons. Thus, unlike cholecystokinin, PYY_{3-36}-induced satiety is atypical, because it does not produce detectable activation of brainstem satiety centers and is not dependent on MC4-R signaling.

INTRODUCTION

Two endogenous forms of peptide YY (PYY_{1-36} and PYY_{3-36}) are synthesized by the gastrointestinal (GI) tract (Pedersen-Bjergaard et al., 1996) and released into the circulation after a meal (Adrian et al., 1985). They are released such that approximately 60% is PYY_{1-36} and the rest is PYY_{3-36} (Raposinho et al., 1999). Both peptides have a number of local effects on the GI system (Bottcher et al., 1993; Pappas et al., 1985) and have orexigenic actions when administered centrally (Marsh et al., 1999a; Morley et al., 1985). Extensive studies of the effects of PYY_{1-36} on food intake with respect to its site of action have shown a differential effect on its ability to increase food intake; intracerebroventricular injections into the fourth ventricle have a much greater effect of stimulating food intake compared with third ventricle PYY_{1-36} administration (Hagan, 2002). In agreement with these studies, reports have shown that PYY_{1-36} predominantly exerts its orexigenic effects via the brainstem (Hagan, 2002). However, elevated systemic levels of PYY_{1-36}, due to a gastric bypass surgery or peripheral injections, have emetic effects, leading to a reduction of food intake (Harding and McDonald, 1989; Naslund et al., 1997).

The PYY_{3-36} form appears to be anorexigenic when given peripherally (Batterham et al., 2002; Challis et al., 2003). In a recent study Batterham and co-workers (Batterham et al., 2002) showed that ip injections of PYY_{3-36}, acting through Y2 receptors, can suppress fast-induced feeding in rats and mice. Additionally, PYY_{3-36} was shown to activate proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (ARC)

when bath-applied to hypothalamic slices in vitro (Batterham et al., 2002). However, a number of laboratories (Thone-Reineke) have had difficulty reliably and reproducibly repeating the anorexigenic effect of PYY_{3-36} in the rodent. One model for the anorexigenic action of PYY_{3-36} proposes activation of ARC POMC neurons of the central melanocortin system (Batterham et al., 2002), and indeed, PYY_{3-36} administration has been demonstrated to elevate hypothalamic POMC mRNA levels (Challis et al., 2003); however, we were concerned by the very low percentage (20% with PYY_{3-36} vs. 8% with saline) of POMC ARC neurons activated by PYY_{3-36}, as assessed by c-Fos immunohistochemistry. In this study we demonstrate a reproducible protocol for assessing the anorexigenic activity of PYY_{3-36} utilizing a long acclimatization of animals, and that the peptide retains full activity in the melanocortin-4 receptor knockout (MC4-$R^{-/-}$) mouse.

MATERIALS AND METHODS

AnimalsMC4-$R^{-/-}$ and POMC-enhanced green fluorescent protein (EGFP) mice were derived from the animals described previously (Cowley et al., 2001a; Huszar et al., 1997) and were bred 10 generations into the C57BL/6J background. All transgenic animals were raised in group housing with their siblings and maintained at 23 ± 1 C on a 12-h light, 12-h dark cycle (0700–1900 h light). Mice were allowed ad libitum access to standard chow pellets (Purina Laboratory Rodent Diet 5001, Ralston Purina Co., St. Louis, MO; 4.5% fat). Wild-type (WT) controls of the C57BL/6J strain were purchased to be age, sex, and weight matched (The Jackson Laboratory, Bar Harbor, ME). Upon

arrival WT mice were allowed to acclimate for 1 wk under the conditions stated above. All studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the animal care and use committee of Oregon Health and Science University.

Source of reagents/peptides

All experiments, unless stated otherwise, were performed with human PYY_{3-36} purchased from American Peptides (Sunnyvale, CA; first batch: lot Q08111T1). A second human PYY_{3-36} batch and the synthetic MC3-R/MC4-R antagonist SHU9119 were purchased form Bachem (Torrance, CA; lot 0558311), and a second independent batch of human PYY_{3-36} was purchased from American Peptides (lot R05026T1). All peptides were certified by the manufacturer and came with HPLC data showing a single peak with the correct molecular weight from mass spectrograms and a purity greater than 97%. Peptides were dissolved in sterile isotonic saline and injected ip in a total volume of 500 μl/injection. Fresh human PYY_{3-36} and SHU9119 concentrations were prepared on the day of injection from frozen stock solutions.

Feeding protocols

Response of unacclimated animals to a 16-h fast.

Age-matched WT (The Jackson Laboratory) male mice (8 wk old) were used for unacclimated feeding studies. Mice were individually housed the day before a nocturnal fast (1800–1000 h). Injections and food consumption measurements were performed in a

double-blinded experiment. Animals were injected ip at 1000 h with either saline or PYY_{3-36} at a dose of 0.3, 3, or 10 μg/100 g. Food intake was measured hourly for the first 4 h and at 12 h, by placing two pellets of chow in petri dishes at the bottom of the cage at the time of ip injection. To minimize error attributable to loss of food particles, all bedding was screened before and after the experiment to capture any spilled food. Food in petri dishes was also screened to remove any bedding or other debris.

Response of acclimated animals to a 16-h fast.

Age-matched WT (The Jackson Laboratory) male mice (8 wk) were used for the feeding study. Mice were individually housed for 1 wk. In the following week they were acclimated to daily ip saline injections at 1000 h with two pellets of food being placed in a petri dish on the floor of the cage and weighed hourly for 4 h. Animals were fasted for 16 h the night before the experiment (1800–1000 h). Food intake was measured by placing two pellets of chow in petri dishes on the floor of the cage at the time of ip injection (double-blinded) of either saline or PYY_{3-36} at a dose of 0.3, 3, or 10 μg/100 g, and cumulative food intake was measured hourly for 4 h post injection. As before, to minimize error attributable to loss of food particles, all bedding and petri dishes were screened.

Response of acclimated animals in a nighttime feeding protocol.

Age-matched MC4-$R^{-/-}$ and WT (The Jackson Laboratory) male mice (8 wk old) were used for the nocturnal feeding study. For habituation, mice were individually housed for 1 wk and injected with daily (500 μl) saline immediately before lights out (1900 h). Two

pellets of food were placed in a petri dish on the floor of the cage immediately after injection, and food intake was measured hourly for 4 h. Animals were habituated until their food intake stabilized for at least 4 consecutive days before experimental treatment. Animals were randomly injected with either saline or 0.3 μg/100 g $PYY_{3–36}$ on the first experimental day, with 3 μg/100 g on the second day, and with 10 μg/100 g on the last experimental day. On nonexperimental days animals were injected with saline to measure deviation from previous habituation baseline. Measurement error was minimized with careful screening of petri dishes for debris and cage bedding for spilled food.

c-Fos immunohistochemistry

POMC-EGFP mice (23–27 g), a transgenic strain in which EGFP is expressed under the control of the POMC promoter (Cowley et al., 2001a), were handled and received 100 μl sterile saline, ip, at 0900 h for 5 d before the experiment to minimize background c-Fos immunoreactivity caused by stress. Animals received an ip injection of $PYY_{3–36}$ (5 μg/100 g) or sterile saline 90 min before being deeply anesthetized and undergoing transcardial perfusion with 0.9% heparinized saline, followed by 4% paraformaldehyde in 0.01 M PBS. Sections were cut at 30 μm from perfused brains and stored free-floating in 0.01 M PBS containing 0.03% sodium azide. The sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS and 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-c-Fos antibody (sc-052, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:6000 in blocking reagent for 24 h at 4 C, followed by incubation in 1:500 donkey anti-rabbit Alexa 594 (Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature. In the

nucleus tractus solitarius (NTS) sections, the POMC-EGFP cells were detected using a 1:4000 dilution of rabbit anti-GFP antibody directly conjugated to Alexa 488 (Molecular Probes, Inc.). Between each stage the sections were washed thoroughly with 0.01 M PBS. At the end of the incubations the sections were mounted onto gelatin-coated slides, coverslipped using gel-based fluorescence mounting medium (Biomeda Corp., Foster City, CA), and viewed under a fluorescence microscope (Axioplan 2, Zeiss, Inc., Thornwood, NY). The number of c-Fos-immunoreactive cells was counted on sections that also contained POMC-EGFP cells by a person blinded to the individual treatments.

Statistics

Statistical analyses were performed using PRISM (GraphPad, San Diego, CA). Data are expressed as the mean ± SEM. One-way ANOVA with Dunnett's test post hoc test was used to determine significance in Fig. 1. Significance was determined using an unpaired (two-tailed) t test in Figs. 2 and 3. Significance was taken as $P < 0.05$.

RESULTS

$PYY_{3\text{–}36}$ does not reduce food intake after a single ip injection in 16-h fasted naive WT mice

We postulated that the inability of some laboratories to reduce food intake with a single ip injection of $PYY_{3\text{–}36}$ in mice (Thone-Reineke) could be due to stress caused by the

experimental procedure (De Souza et al., 2000). To test this hypothesis we individually housed naive/unacclimated WT male mice at 1700 h and fasted them for 16 h (1800–1000 h) before a single ip injection of either saline or 0.3, 3, or 10 μg/100 g $PYY_{3–36}$ in a double-blinded experiment. $PYY_{3–36}$ did not reduce food intake at any dose or any time point (Fig. 1A). Unacclimated WT saline controls (Fig. 1A) ate 32.0% (Fig. 1B) and 53.5% (Fig. 1, C and D) less than acclimated WT saline controls in the first hour of measurement [unacclimated, 0.48 ± 0.13 g (Fig. 1A); acclimated, 0.71 ± 0.04 g (Fig. 1B); acclimated, 1.04 ± 0.08 g (Fig. 1, C and D)].

$PYY_{3–36}$ reduces food intake in a dose-dependent manner after a single ip injection after 16-h fast in acclimated mice

Due to our previous experience we tested $PYY_{3–36}$ actions after a week-long acclimatization protocol that we have shown to reduce stress due to handling (De Souza et al., 2000). In this study WT animals were individually housed for 1 wk and treated daily with ip saline injections, and food was weighed at the appropriate times for acclimation. The animals were fasted for 16 h before ip injection of $PYY_{3–36}$ at varying doses of 0.3, 3, and 10 μg/100 g, and cumulative food intake was measured at 1-h intervals for 4 h. Compound administration and food intake measurements were performed using a double-blind procedure to prevent any handling artifacts or experimental bias. $PYY_{3–36}$ reduced food intake in a dose-dependent matter in the first 4 h after administration (Fig. 1B). $PYY_{3–36}$ significantly reduced food intake in the 3 and 10 μg/100 g treatment groups compared with the saline-treated animals at both 1 and 2 h

(Fig. 1B). Although there was a trend toward a reduction in food intake by PYY_{3-36} with the 0.3 μg/100 g dose, statistical significance was not reached (Fig. 1B). Four hours after the ip injections, only the 10 μg/100 g dose retained its ability to significantly reduce food consumption (Fig. 1B). At 12 h post injection, no difference in food intake was measured at any concentrations (Fig. 1B, inset).

PYY_{3-36} from different batches and companies reliably reduces food intake after a 16-h fast in acclimated WT mice in a dose-dependent manner

Given the small degree of anorexia caused by PYY_{3-36} and the limited duration of its action, we postulated that negative results might be due to batch to batch differences in the peptide. We next tested the effects of two different batches of PYY_{3-36} obtained from two separate manufacturers on reducing food intake by a double-blinded experiment in 16-h fasted WT mice acclimated to handling. Peptides from both companies reduced food intake in habituated animals in a dose-dependent fashion (Fig. 1, C and D) Bachem PYY_{3-36} showed a dose-response curve with increasing doses of peptide (Fig. 1D), whereas American Peptides PYY_{3-36} showed a saturation of its effects past the 3 μg/100 g dose (Fig. 1C). Additionally, PYY_{3-36} from Bachem also acted in a short-term manner, where it reduced food intake significantly only for the first 2 h after injection (Fig. 1D) like American Peptides PYY_{3-36} in Fig. 1B. As before (Fig. 1B), both new batches of peptides were unable to reduce food intake at the lowest dose (0.3 μg/100 g) at any time point measured, and, in fact, at the single time point of 3 h American Peptides PYY_{3-36}

stimulated feeding (Fig. 1C). At 10 μg/100 g, peptides from both companies significantly reduced food intake for the duration of the experiment (Fig. 1, C and D).

It is possible that the reduction in food intake that was observed in the previous experiments could have simply been due to ip injection of any peptide or from a nonspecific compound that the particular company uses for its peptide storage. To test this hypothesis we obtained the MC3/4R antagonist SHU9119 from Bachem simultaneously with PYY_{3-36}. The ip injections of SHU9119 (3 and 10 μg/100 g) in a double-blinded experiment performed in 16-h fasted WT acclimated mice had no effect on food intake at any time point that PYY_{3-36} dose-dependently reduced food intake (data not shown).

MC4-R is not required for inhibition of feeding by PYY_{3-36}

PYY_{3-36} increases POMC neuronal firing in hypothalamic slices (Batterham et al., 2002) and POMC mRNA expression acutely after peripheral PYY_{3-36} administration (Challis et al., 2003). However, ip injection of PYY_{3-36} only increases the expression of c-Fos, an indirect marker of neuronal activation, in approximately 12% of ARC POMC neurons (Batterham et al., 2002). These results suggested that the role of the melanocortin system in the action of PYY_{3-36} needed to be examined more carefully. To test the role of the melanocortin system in the action of PYY_{3-36}, we evaluated the ability of MC4-$R^{-/-}$ mice to show an anorexigenic response after ip administration of PYY_{3-36}. We chose a nocturnal feeding response to PYY_{3-36} because it is most physiologically relevant. WT

and MC4-R$^{-/-}$ feeding ad libitum were injected with either saline or PYY_{3-36} at increasing doses immediately before lights out (1900 h), and cumulative food intake was measured hourly for 4 h. The freely night-feeding WT animals showed a similar response to increasing doses of PYY_{3-36} as fasted animals (Fig. 1). At the lowest dose, PYY_{3-36} (0.3 μg/100 g) did not significantly reduce nocturnal food intake in WT mice over the 4 h of measurement (Fig. 2A). At the 3 μg/100 g dose, PYY_{3-36} reduced food intake significantly in WT over the first 3 h (Fig. 2B). At the highest dose of 10 μg/100 g, PYY_{3-36} significantly reduced food intake in WT for the 4 h of measurements (Fig. 2C).

MC4-R$^{-/-}$ mice injected with PYY_{3-36} exhibited a similar dose-dependent decrease in ad libitum nocturnal food intake as WT animals (Fig. 2). Like WT animals, the lowest dose of PYY_{3-36} (0.3 μg/100 g) did not induce a significant decrease in food intake in MC4-R$^{-/-}$ mice at any of the time points measured (Fig. 2A). The intermediate dose of PYY_{3-36} (3 μg/100 g) transiently reduced food intake in MC4-R$^{-/-}$ mice to a similar degree and over the same time course as in WT mice (Fig. 2B). At the highest dose of 10 μg/100 g PYY_{3-36}, MC4-R$^{-/-}$ animals responded in the same way as WT mice (Fig. 2C).

Daytime peripheral PYY_{3-36} administration induces c-Fos expression in POMC neurons in the ARC

To further examine the possible role of POMC neurons in mediating the effects of PYY_{3-36}, we performed a preliminary experiment to examine the activation of POMC neurons in both the ARC and NTS using c-Fos immunohistochemistry. Immunohistochemical

experiments were performed using a previously characterized transgenic mouse in which EGFP is expressed under the control of the POMC promoter (Cowley et al., 2001a); thus, EGFP immunoreactivity was used to visualize POMC-positive cells. Daytime ip injection at 0900 h of PYY_{3-36} at 5 μg/100 g significantly increased c-Fos expression in POMC neurons in the ARC from 9% to 22% (Fig. 3A) similar to data reported previously (Batterham et al., 2002). No increase was seen in c-Fos-positive POMC neurons in the NTS; however, this analysis involved a small sample (three animals) examined under a single condition (Fig. 3C). In this preliminary experiment there was a trend upward, but no significant increase in total c-Fos expression in the ARC (Fig. 3B) or NTS (Fig. 3D) was found compared with saline-treated animals.

DISCUSSION

In this study we examined the effects of PYY_{3-36} on food intake in mice during daytime feeding after a 16-h fast and during normal nighttime feeding. We show here that in both paradigms PYY_{3-36} dose-dependently reduces food intake in acclimated animals. Furthermore, the degree and duration of food reduction are similar at the same doses of PYY_{3-36} administered from different batches, manufacturers, and species of peptide. At the lowest dose of peptide (0.3 μg/100 g), a trend in food reduction was seen in the first and second hours of measurement, which did not reach statistical significance. At the higher dose of the peptide (3 μg/100 g), a significant reduction in food intake was observed during the first 2 h in the daytime experiments and during the first 3 h of the nocturnal experiment. However, the inhibition of food intake by PYY_{3-36} was short-lived.

At the dose of 3 μg/100 g, the effect of $PYY_{3–36}$ was reversed by a rebound hyperphagia by the fourth hour post injection, as indicated by the loss of significance in cumulative food intake between the second and third hours in daytime experiments and between the third and fourth hours in the nocturnal experiments. This trend was further observed at the highest dose of $PYY_{3–36}$ (10 μg/100 g), but the reduction in food intake at this dose remained significant for the duration of the experiment. By 12 h post injection, no difference in food intake was seen between treatments at any concentration studied in the daytime 16-h fasted experiment. The results of these experiments would argue that $PYY_{3–36}$ has a similar mechanism of action in its ability to reduce food intake after a fast or during physiologically relevant nocturnal feeding. The short duration of action of $PYY_{3–36}$ and its inability to reduce 12-h food intake are reminiscent of the action of the satiety factors cholecystokinin (CCK) and bombesin (Crawley and Beinfeld, 1983; Crawley and Corwin, 1994).

Recently, there have been reports that $PYY_{3–36}$ is incapable of reliably reducing food intake (Thone-Reineke). Here we showed that without proper habituation of the WT mice, the satiating effect of $PYY_{3–36}$ was not evident. This loss of efficacy may be due to the effects of stress caused by handling and ip injection, as evident by the 32% decrease in food intake in unacclimated WT mice compared with acclimated WT mice (De Souza et al., 2000). Therefore, as $PYY_{3–36}$ has a modest anorexigenic efficacy and a short time window of action, its effects may be insignificant relative to the prominent reduction in food intake due to stress. Alternatively, if $PYY_{3–36}$ and stress activate common anorexigenic circuits, stress-induced anorexia could mask the effects of $PYY_{3–36}$. This,

however, is an unlikely since we found that neither the central nor peripheral corticotropin releasing factor (CRF) receptors, CRF_1 and CRF_2 receptors, known to mediate the short (Hotta et al., 1999; Tache et al., 2001) and long-term (Reyes et al., 2001; Wang et al., 2001) inhibition of food intake, respectively, are required for the anorexigenic actions of PYY_{3-36} (Appendix Fig. 3 and 4).

The PYY_{3-36}-induced reduction in food intake is small even at high doses, and the effect is very short-lived. Thus, the action of PYY_{3-36} could also be easily missed if food intake is not measured within the first 4 h after injection or if the peptide is administered sc vs. ip. For example, a study by Challis et al. (Challis et al., 2003) showed that PYY_{3-36} (10 μg/100 g) reduced food intake after a 24-h fast, but did not measurably reduce nocturnal food intake in nonfasted, freely feeding mice when food intake was measured 6 h post injection. The lack of response seen with this paradigm may be due to the fact that food intake was not measured until 6 h after peptide administration. In our study the effect of PYY_{3-36} on food intake was largely absent by this time point. Furthermore, it is not clear whether these animals were acclimated to handling before the procedure, but this may be a contributing factor to the lack of response seen.

Additionally, peptides from different species, rat and human (Appendix Fig. 1), and companies, American Peptides and Bachem, both similarly and reliably reduced fast-induced food intake in the first 4 h of measurement in a double-blinded experiment, which was used to prevent any handling artifacts.

In this study we further tested the hypothesis that PYY_{3-36} acts via the central melanocortin system. The original report by Batterham and colleagues (Batterham et al., 2002) showing that PYY_{3-36} has anorexigenic effects in mice also proposed that these effects might be mediated by the central melanocortin system. They observed an increase in POMC neuron firing with PYY_{3-36} in an electrophysiological slice preparation and an increase in c-Fos expression in POMC neurons of the ARC. We wanted to further examine this model in two ways: 1) by examining whether the MC4-R is essential for the anorexigenic action of PYY_{3-36}, and 2) by addressing whether POMC neurons in the ARC are activated by PYY_{3-36} and whether a potent activation of brain stem neurons by PYY_{3-36} is seen, as is the case with CCK or gastric distention (Cano et al., 2003; Traub et al., 1996; Wang et al., 1998; Zittel et al., 1999). Here we show that MC4-$R^{-/-}$ mice are just as responsive to the anorexigenic effects PYY_{3-36} as WT mice in a nocturnal feeding paradigm. This result argues that the MC4-R is not essential for the anorexigenic action of PYY_{3-36}. Therefore, although the increase in the POMC neuron firing rate in slice preparations (Batterham et al., 2002) and the induction of POMC mRNA (Challis et al., 2003) by PYY_{3-36} may indeed lead to an increase in melanocortin signaling though the MC4-R, this does not appear to be essential for the anorexigenic effects of the peptide. Furthermore, we also show that MC3-$R^{-/-}$ mice are just as responsive to the anorexigenic effects of PYY_{3-36} as MC3-$R^{+/+}$ mice in 16 h fast-induced refeeding paradigm (Appendix Fig. 2), indicating that neither the MC3-R nor the MC4-R are required for the anorexigenic actions of PYY_{3-36}.

In our preliminary immunohistochemical study, a small, but significant, increase in of c-Fos was seen in ARC POMC neurons after daytime administration of PYY_{3-36}, consistent with previously published results (Batterham et al., 2002). Surprisingly, only a small nonsignificant increase in c-Fos immunoreactive positive cells was observed in total NTS neurons and NTS POMC neurons. The relatively small increase in c-Fos expression in the ARC and the lack of a significant increase in NTS may be due to a number of factors. First, the actions of PYY_{3-36} via the autoinhibitory Y2 receptor may indirectly modulate anorexigenic neurons and thus may not be potent enough to activate c-Fos; for example, in the ARC PYY_{3-36} appears to stimulate POMC neurons by indirectly decreasing the inhibitory -aminobutyric acid-ergic drive onto POMC neurons. Alternatively, PYY_{3-36} may inhibit feeding via a mechanism quite distinct from other gut-derived satiety factors. The lack of significant c-Fos expression in the NTS after PYY_{3-36} administration is in contrast to other GI peptides such as CCK and bombesin (Bonaz et al., 1993; Fraser and Davison, 1992), which also have short-term satiety-like actions yet induce c-Fos immunoreactivity in a great number of NTS neurons (Bonaz et al., 1993; Fraser and Davison, 1992). We have recently demonstrated that ip CCK routinely activates greater than 30% of POMC NTS neurons under similar experimental conditions, and furthermore, that the anorexic actions of CCK are blocked by either genetic or pharmacologic MC4-R blockade (Fan et al., 2004). Regardless of the causes behind the limited up-regulation of c-Fos in NTS and ARC after inhibition of feeding by PYY_{3-36}, the data presented here argue that PYY_{3-36} inhibits food intake via a strikingly different mechanism than that of either the long-term adipostatic factor leptin, which can potently

activate ARC POMC neurons (Elias et al., 1999), or satiety signals, such as CCK, bombesin, or gastric distension, which potently activate c-Fos in NTS neurons.

In summary, we have presented data indicating that the satiating effect of PYY_{3-36} is complex, atypical, and does not require the presence of the MC3/4-R. If the MC3/4-R is not essential, other mechanisms must be involved. They may include atypical mechanisms of action of PYY_{3-36} on POMC neurons, other sites of action of PYY_{3-36} in the central nervous system, or, finally, a possible peripheral mode of action.

ACKNOWLEDGMENTS

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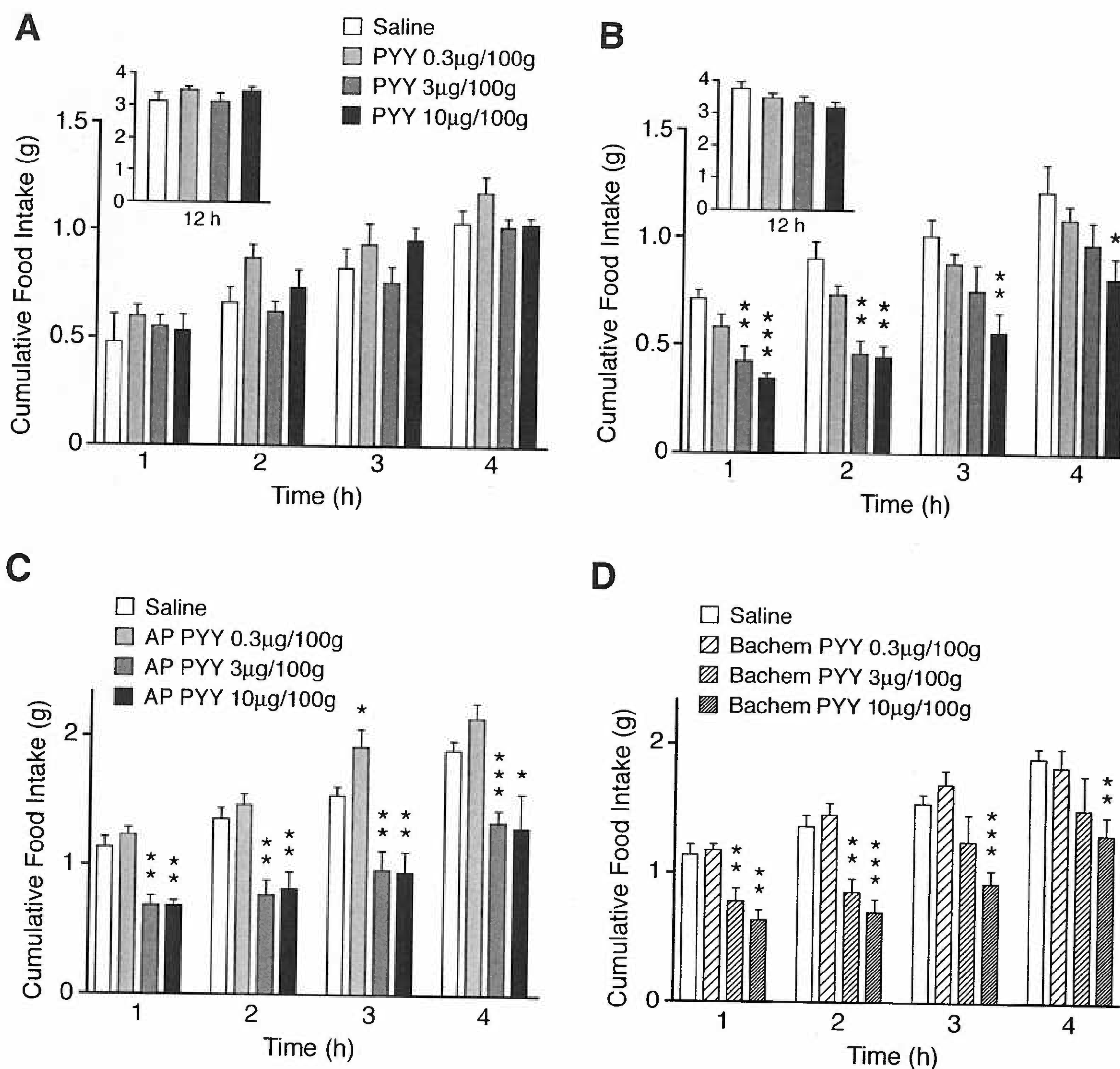

Fig. 1. PYY_{3-36} reproducibly inhibits food intake in a dose-dependent manner after a 16-h fast in acclimated WT mice. A, Feeding response to increasing doses of PYY_{3-36} at 1, 2, 3, 4, and 12 h after ip injection in unacclimated WT mice (n = 5). B, Dose-dependent inhibition of food intake at 1, 2, 3, and 4 h after ip PYY_{3-36} injection in WT mice acclimated for 1 wk (n = 5). No effect of PYY_{3-36} is seen at 12 h after ip injection (*inset*). C, Dose-dependent inhibition of food intake in acclimated WT mice with PYY_{3-36} (American Peptides, batch 2) at 1, 2, 3, and 4 h after ip injection (saline, n = 10; 0.3 μg/100 g, n = 5; 10 μg/100 g, n = 4). D, Dose-dependent inhibition of food intake in acclimated WT mice with PYY_{3-36} (Bachem) at 1, 2, 3, and 4 h after ip injection (saline, n = 10; 3 μg/100 g, n = 4; 10 μg/100 g, n = 5). Data are expressed as the mean ± SEM. By one-way ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

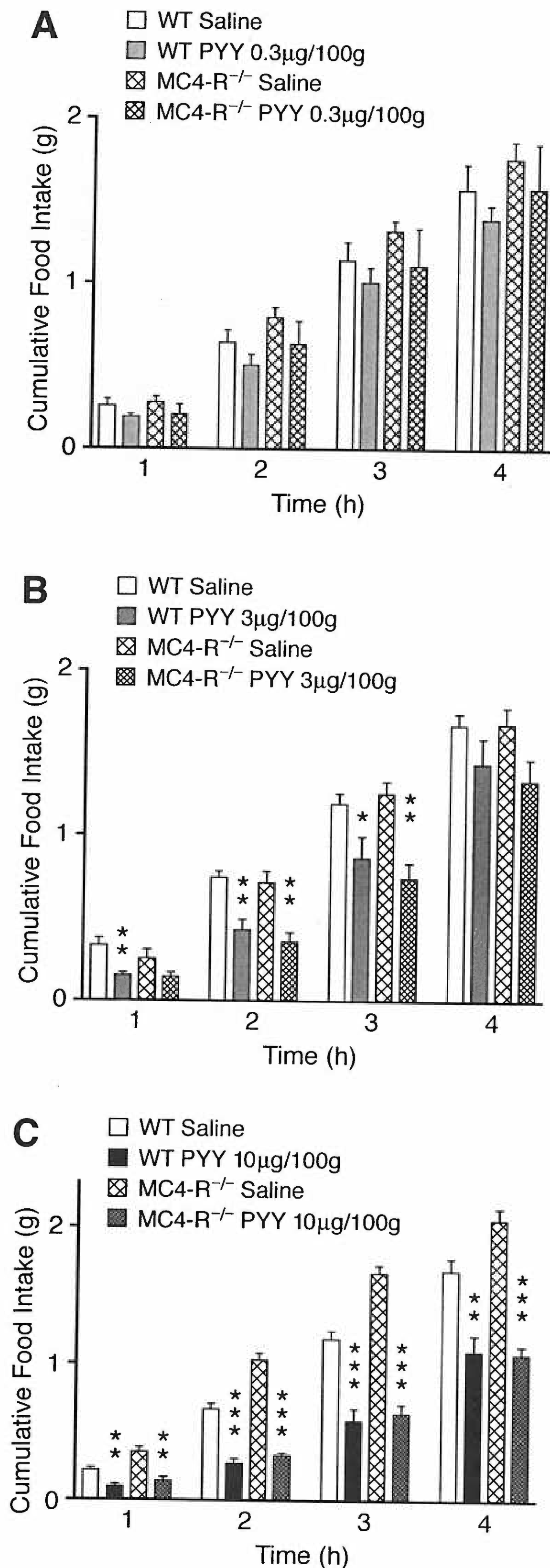

Fig. 2. MC4-R$^{-/-}$ mice and WT mice respond equivalently to increasing concentrations of PYY_{3-36} in a nocturnal feeding paradigm. A, Nocturnal feeding responses of WT and MC4-R$^{-/-}$ mice to a PYY_{3-36} dose of 0.3 μg/100 g at 1, 2, 3, and 4 h post injection (WT saline and 0.3 μg/100 g, n = 6; MC4-R$^{-/-}$ saline, n = 6; 0.3 μg/100 g, n = 5;). B, Nocturnal feeding responses of WT and MC4-R$^{-/-}$ mice to a PYY_{3-36} dose of 3 μg/100 g at 1, 2, 3, and 4 h post injection (WT saline, n = 6; 3 μg/100 g, n = 5; MC4-R$^{-/-}$ saline and 3 μg/100 g, n = 6). C, Nocturnal feeding responses of WT and MC4-R$^{-/-}$ mice to a PYY_{3-36} dose of 10 μg/100 g at 1, 2, 3, and 4 h post injection (all animals, n = 6). Data are expressed as the mean ± SEM. By two-tailed *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

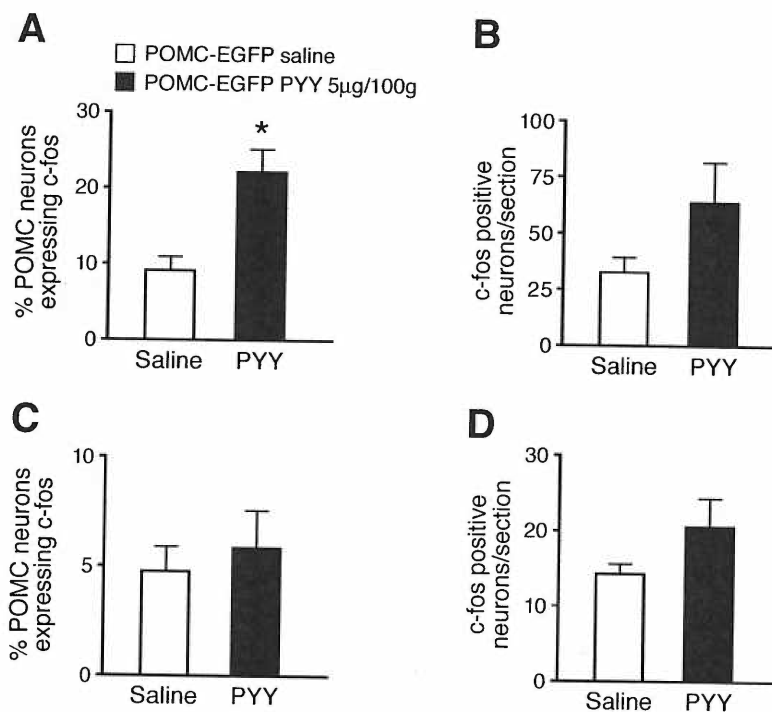

Fig. 3. PYY_{3-36} at dose of 5 µg/100 g activates POMC neurons after 90 min in the ARC, but not in the NTS of the brainstem. A, Approximately 22% of POMC neurons of the ARC are activated by ip administration of PYY_{3-36} compared with 9% by saline. B, There was no significant increase in c-Fos expression in total ARC after ip PYY_{3-36} treatment. C, POMC neurons of the NTS were not activated by PYY_{3-36} treatment. D, There was no significant increase in c-Fos expression in NTS neurons after ip administration of PYY_{3-36}. Saline, n = 4; 5 µg/100 g, n = 3. Data are expressed as the mean ± SEM. By two-tailed *t* test: *, $P < 0.05$.

CHAPTER TWO

Peripheral administration of PYY_{3-36} produces conditioned taste aversion in mice

Ilia G. Halatchev and Roger D. Cone

ABSTRACT

Peptide YY (PYY) is a postprandially released gut hormone. Peripheral administration of one form of the peptide PYY_{3-36} produces a short-term reduction in food intake in rodents. Initial reports suggested that effects of PYY_{3-36} on food intake are mediated by increasing the anorexigenic drive from melanocortin neurons in the hypothalamic arcuate nucleus. However, more recent data have demonstrated that the anorexigenic activity of PYY_{3-36} is not dependent on melanocortin ligands or their receptors in the CNS. We demonstrate here that the anorexigenic actions of PYY_{3-36} are also not dependent on the vagus nerve, a common pathway of satiety signaling. Peripherally administered PYY_{3-36} activates neurons in the area postrema and nucleus tractus solitarius, brainstem areas known to mediate effects of certain aversive stimuli. Furthermore, peripheral administration of PYY_{3-36} causes conditioned taste aversion in mice. Thus, inhibition of food intake by PYY_{3-36} may result in part from induction of an aversive response.

INTRODUCTION

Key sites in the brain, like the arcuate nucleus (ARC) of the hypothalamus and the nucleus tractus solitarius (NTS) of the brainstem, maintain energy homeostasis by integrating peripheral signals of energy availability with energy intake and expenditure. The energy normally used for expenditure comes in two forms: either as stored energy, in the form of glycogen and fat deposits, or as newly ingested energy from a meal. Leptin, an adipostatic signal, is known to relay information about the long-term energy reserves in adipocytes. Gut peptides, like cholecystokinin (CCK) and ghrelin, along with vagal afferent signals, relay satiety and hunger signals to regulate meal size and frequency.

CCK is the archetypal satiety peptide released postprandially from the gut. The proposed mechanism by which CCK causes satiety, under physiological conditions, is through activation of CCK-A receptors on the vagus (Ritter and Ladenheim, 1985). After activation, vagal afferents transmit the signal to the NTS, activating a broad array of NTS neurons to stimulate meal termination. However, at high concentrations, peripherally administered CCK causes vagally mediated taste aversion (Ervin et al., 1995; Verbalis et al., 1986) and activates neurons in the area postrema (AP) (Luckman, 1992), a circumventricular organ outside the blood-brain barrier (BBB) implicated in mediating the response to aversive stimuli (Miller and Leslie, 1994).

PYY$_{3\text{-}36}$ is yet another secreted gut peptide, first reported by two laboratories, to produce a short-lived inhibition of food intake in rats and mice and to reduce the rate of weight

gain in rats (Batterham et al., 2002). This peptide has achieved additional attention due to the observation that continuous intravenous infusion of PYY_{3-36} reduced 24 h food intake and hunger scores in a small number of lean and obese human subjects, suggesting a potential clinical utility of the peptide in the treatment of obesity (Batterham et al., 2003; Batterham et al., 2002). However, following the initial report, 12 laboratories, in a single publication, reported a lack of anorexigenic activity for PYY_{3-36} in rats and mice and also did not find a reduction in the rate of weight gain (Tschop et al., 2004). Since that time, five additional laboratories have repeated the original finding of acute inhibition of feeding by peripheral administration of PYY_{3-36} in rats or mice (Adams et al., 2004; Challis et al., 2004; Chelikani et al., 2005; Cox and Randich, 2004; Pittner et al., 2004), and one laboratory has demonstrated inhibition of feeding in rhesus monkeys (Moran et al., 2005). Two additional observations may shed light on this discrepancy. First, careful acclimatization of mice appears to be required to see the effects of PYY_{3-36} on acute food intake (Halatchev et al., 2004), suggesting that the effect of the peptide may be sensitive to a variety of stressors. Secondly, intravenous infusion of PYY_{3-36} in rats for 3 h, meant to mimic the postprandial rise in the peptide, potently inhibited food intake, while a 15 min dosing was much less efficacious, suggesting that the bolus intraperitoneal (ip) administration of the peptide used in most studies may be a much less effective route of administration (Chelikani et al., 2005). Indeed, 11 of the 12 studies in the negative report utilized ip administration; the single study using subcutaneous infusion showed a statistically significant inhibition of food intake for 3 days (Tschop et al., 2004). One study using subcutaneous infusion of the peptide has now reproduced the initial finding

of a reduction of cumulative food intake and the rate of weight gain in a variety of normal and obese murine models (Pittner et al., 2004).

PYY_{1-36} is secreted from L cells in the ileum and colon in response to ingested fatty acids and other nutrients (Adrian et al., 1985; Bottcher et al., 1986; Bottcher et al., 1993). In the circulation, PYY_{1-36} is cleaved by dipeptidyl peptidase-IV (DPP-IV) to produce PYY_{3-36} (Grandt et al., 1994b). The PP fold, a common tertiary structure shared between PYY_{3-36} and all other NPY family peptides, confers its specificity for the NPY receptors 1–5 (Y1–Y5) (for review, see (Keire et al., 2002)). PYY_{3-36} has high-affinity binding restricted to the Y2 (Ki = 0.03–0.3 nM) and Y5 receptors (Keire et al., 2002).

Initially, PYY_{3-36} was postulated to exert its anorexigenic effects through activation of ARC POMC neurons (Batterham et al., 2002). Using an in vitro slice preparation, PYY_{3-36} was observed to reduce inhibitory postsynaptic potentials onto POMC neurons and increase the basal firing rate of these neurons, presumably via Y2 receptor on NPY neurons (Batterham et al., 2002). In this model, PYY_{3-36} is proposed to have direct anorexigenic actions in the ARC, gaining access either through the median eminence, a circumventricular organ, or through the blood-brain barrier. Furthermore, peripheral administration of PYY_{3-36} increased c-Fos immunoreactivity (IR), a marker of neuronal activation, in ~12% of POMC neurons in the ARC, compared to saline-treated mice (Batterham et al., 2002; Halatchev et al., 2004).

However, a number of recent reports have shown that this model may need to be reconsidered. Unexpectedly, in a nocturnal feeding and/or fast-induced refeeding paradigm, MC4-R knockout (MC4-R$^{-/-}$) (Halatchev et al., 2004), MC3-R knockout (MC3-R$^{-/-}$) (Appendix Fig. 2), Agouti (Martin et al., 2004), and POMC knockout mice (POMC$^{-/-}$) (Challis et al., 2004) showed a reduction of food intake comparable to wild-type (wt) sex- and age-matched controls after peripheral administration of PYY_{3-36}. These studies demonstrated that melanocortin signaling is not necessary for the anorexigenic effects of PYY_{3-36}, and an alternative mechanism should be considered.

In this study, we sought to identify alternative mechanisms of action for PYY_{3-36}. We first tested if, like CCK, PYY_{3-36} requires the vagus nerve for its actions or requires intact leptin signaling. Using c-Fos as a marker of neuronal activation, we also systematically examined brain regions activated by increasing peripheral doses of PYY_{3-36} in order to identify other potential sites of action. Finally, we examined the ability of the peptide, at doses that reliably and reproducibly inhibit food intake, to induce conditioned taste aversion.

MATERIALS AND METHODS

Animals

Transgenic mice (C57BL/6J background) with enhanced green fluorescent protein under the control of the POMC promoter (EGFP-POMC) were derived from animals described previously (Cowley et al., 2001b). All transgenic animals were raised in group housing

with their siblings and maintained at 23°C ± 1°C on a 12 h light, 12 h dark cycle (0700–1900 h light). Mice were allowed ad libitum access to standard chow pellets (Purina Laboratory Rodent Diet 5001, Ralston Purina Co., St. Louis, MO; ~4.5% fat by weight). $Lepr^{db}/Lepr^{db}$ and wt experimental animals of the C57BL/6J strain were purchased to be age, sex, and weight matched (The Jackson Laboratory, Bar Harbor, ME). Lep^{ob}/Lep^{ob} mice, male and female, were purchased from the same source to be age and weight matched. BSDV male mice (C57BL/6J strain) were purchased with both the anterior and posterior trunks of the vagus nerves cut below the level of the diaphragm (Charles River Laboratories, Wilmington, MA). To create Sham-operated animals, C57BL/6J mice from the same source underwent a comparable surgery without transection of the vagus nerves. Upon arrival, Lep^{ob}/Lep^{ob}, $Lepr^{db}/Lepr^{db}$, BSDV, Sham, and wt mice were allowed to acclimate for 1 week under the conditions stated above. All studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the animal care and use committee of Oregon Health and Science University.

Source of reagents/peptides

All experiments were performed with human PYY_{3-36} (Bachem, Torrance, CA; lot 0562660), CCK-8 (Sigma-Aldrich, St. Louis, MO; lot 062K11181), and LiCl (Sigma-Aldrich, St. Louis, MO; lot 50K0198). Peptides were certified by the manufacturer and came with HPLC data showing a single peak with the correct molecular weight from mass spectrogram and a purity grater than 97%. Peptides were dissolved in sterile isotonic saline and injected in a total volume of 500 μl/injection. Fresh human PYY_{3-36}

and CCK-8 dilutions were prepared on the day of the injection from frozen stock solutions. LiCl was dissolved in sterile millipore-filtered water (0.66 g in 100 ml of water).

Feeding protocols

16 h fast refeeding in acclimated wt mice

Age-matched wt male mice (8 weeks) were used for the feeding study as described previously (Halatchev et al., 2004). In short, animals were individually housed for a week. During the second week, animals were acclimated to the procedure by ip saline injections daily, with food in a petri dish and mock food measurements every hour for 4 h. Mice were fasted for 16 h the night before the experiment (1900–1100 h). Animals were injected with either saline or PYY_{3-36} ip at a dose of 0.3, 1.5, and 5 μg/100 g, and cumulative food intake was measured hourly for 4 h after injections by placing two pellets of chow in petri dishes on the floor of the cage. To minimize error attributable to loss of food particles or bedding in petri dishes, petri dishes and bedding were screened.

Nighttime feeding protocol in acclimated Lep^{ob}/Lep^{ob}, $Lepr^{db}/Lepr^{db}$ mice

Age-matched Lep^{ob}/Lep^{ob} (8- to 9-week-old, half male and half female) and $Lepr^{db}/Lepr^{db}$ (10-week-old female mice) were used for the nocturnal study. As described previously (Halatchev et al., 2004), mice were individually housed for a week. In the following week, mice were acclimated to the procedure by injecting 500 μl sterile isotonic saline ip immediately before lights out (1900 h). Two pellets of food were then placed in a petri

dish and weighed every 2 h for 6 h under a red light. Animals were acclimated to the procedure until their food intake stabilized for at least 4 consecutive days. On the 2 experimental days, separated by 48 h of rest, animals were injected ip in a crossover manner with either saline or 5 μg/100 g $PYY_{3–36}$ and their food intake measured every 2 h for 6 h. On nonexperimental days, animals were injected ip with saline and food measured to establish any deviations from baseline. Measurement error was minimized by careful screening for food particles under red light.

Nighttime feeding protocol in acclimated Sham and BSDV mice

Age-matched Sham and BSDV male mice (8 weeks) were used for the nocturnal vagotomy feeding experiment. Mice underwent surgery a week before arrival and were allowed to recover their body weight and food intake to presurgical levels for a week in group housing. Mice were individually housed for a week, and their body weight was monitored and compared across surgical groups and to age-matched sex-matched nonsurgical mice. Only mice that recovered completely from surgery were used for the experiment. Sham and BSDV mice were injected with 500 μl of sterile isotonic saline ip immediately before lights out (1900 h), and two preweighed pellets of Purina chow in petri dishes were measured every 2 h for 8 h using red light. Acclimatization to the procedure was established by stabilization of food intake over 4 consecutive days. On experimental days, sham and BSDV mice were injected ip in a crossover manner with either saline or 5 μg/100 g $PYY_{3–36}$ and their food intake measured every 2 h for 8 h.

CCK-8, a peptide demonstrated to require the vagus nerve to mediate its effects (Ritter and Ladenheim, 1985), was used to confirm that the BSDV mice were vagotomized and that the Sham-vagotomized animals were not. Animals received 2 days of isotonic saline injections ip after $PYY_{3–36}$ administration to confirm that baseline food consumption was not altered due to experimental manipulations. On the third and fifth days, animals were injected ip in a crossover manner with either saline or CCK-8 (10 μg/kg) and their food intake measured at 2 h. Each animal was used as its own control to determine if its vagus nerves were cut. Only Sham animals that responded to CCK-8 by reducing their food intake and only BSDV mice that did not show a reduction of food intake with CCK-8 were used for analysis of the effect of vagotomy on the actions of $PYY_{3–36}$. For consistency, each individual animal's response to CCK-8 was tested several times.

c-Fos immunohistochemistry

POMC-EGFP mice (23–27 g) were handled and injected with 250 μl sterile saline ip at 1900 h for 5 days prior to the experiment to minimize background c-Fos immunoreactivity caused by stress. During the acclimatization period, food was withdrawn from animals 2 h prior to injections (for 3.5 h) to minimize neuronal activation due to feeding (Fan et al., 2004). Animals received an ip injection of $PYY_{3–36}$ (0.3, 1.5, and 5 μg/100 g), CCK-8 (10 μg/kg), or sterile saline 90 min (at lights out, 1900 h) before being deeply anaesthetized and underwent transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). Sections from perfused brains (25 μM) were cut and stored free-floating in 0.01 M PBS containing 0.03% sodium azide. The sections were incubated for 1 h at room temperature

in blocking reagent (5% normal donkey serum in 0.01 M PBS + 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-c-Fos (Ab-5), human (rabbit) polyclonal antibody (Oncogene Research Products, La Jolla, CA; Lot D21570) and diluted 1:80,000 in blocking reagent for 24 h at 4°C, followed by incubation in 1:500 donkey anti-rabbit Alexa 594 (Molecular Probes Inc., Eugene, OR) for 1 h at room temperature. Between each stage, the sections were washed thoroughly with 0.01 M PBS. At the end of the incubations, the sections were mounted onto gelatin-coated slides, coverslipped using gel-based fluorescence mounting media (Biomeda Corp., Foster City, CA), and viewed under a fluorescence microscope (Axioplan 2; Zeiss Inc., Thornwood, NY). The number of c-Fos immunoreactive cells was counted in sections representing certain nuclei.

Conditioned taste aversion

Age-matched (7-week-old) male wt (C57BL/6J) mice were individually housed for 1 week with ad libitum access to food and water. After 1 week of acclimatization to single housing, water was withdrawn right before lights out (day 0). To acclimate mice to the ip injections, weighing, and timing of water presentation, animals were presented with two water bottles (equidistant from the food, and the original water bottle presented during the first week of individual housing) at the same time (1000 h–1100 h) for 1 h for 5 consecutive days (day 1–day 5). At the end of the hour, water bottles and mice were weighed, and each animal was injected ip with a volume of sterile isotonic saline, equal to 2% of its body weight (BW). Individual water consumption from each bottle was measured for each mouse every day to ensure mice knew that they had a choice between

two bottles and drank equally from both. On day 6, each mouse was given 1 h access to a novel flavor (either 0.15% saccharin with 0.05% cherry Kool-Aid or 0.15% saccharin with 0.05% grape Kool-Aid) instead of water in both bottles. Immediately following the 1 h access to a novel flavor, each mouse was weighed and received ip injection of 2% BW sterile isotonic saline. On the following day (day 7), mice were given access to water for 1 h, weighed, and injected ip with 2% BW saline. On the second Kool-Aid day (day 8), each mouse received a novel access to the other flavor (mice that received cherry on day 6 received grape on day 8, and mice that received grape on day 6 received cherry on day 8) in both bottles, and, immediately following 1 h access to the second novel flavor, each mouse was weighed and injected ip with either 2% BW of saline, 0.15M LiCl, or PYY_{3-36} at doses of 1.5 or 5 μg/100 g. Animals received another day with 1 h water access (day 9) followed by weighing and 2% BW ip saline injection. On the third day of Kool-Aid presentation (day 10), mice were allowed access to 1 h of original Kool-Aid flavor in both bottles (Kool-Aid flavor from day 6) immediately followed by weighing and 2% BW ip saline injection for all animals. After a day with 1 h water access, weighing, and 2% BW ip saline injections (day 11), mice received a fourth day of Kool-Aid access to second flavor (same as day 8) for 1 h in both bottles followed by weighing and ip injection with either 2% BW of saline, 0.15 M LiCl, or PYY_{3-36} at doses of 1.5 or 5 μg/100 g (day 12). Animals were allowed to recover for 48 h by another day of 1 h access to water, weighing, and 2% BW ip saline injections (day 13). On test day, each mouse was presented with both Kool-Aid flavors in random fashion, alternating bottle positions (e.g., left bottle grape versus right bottle grape), simultaneously, and the

respective individual bottle/flavor consumption was measured for 1 h. See Figure 4 for schematic representation of the conditioned taste aversion (CTA) protocol.

The CTA data was represented as raw data (Figure 5A) of the volume consumption by each animal (for the saline group, volume consumed from either right or left bottle and, for stimulus-paired groups, volume consumed of the saline-paired flavor [day 6 and day 10] versus PYY_{3-36}-paired or LiCl-paired flavor [day 8 and day 12]). Additionally, ratios of volume consumed (Figure 5B) were calculated as (volume bottle 1 or 2 consumed)/(total volume consumed) for saline controls or (volume of saline-paired flavor consumed)/(volume of stimulus-paired flavor consumed + volume of saline-paired flavor consumed) for experimental treatments. Even though individual C57BL/6J mice showed some flavor preference (for either grape or cherry), on average no preference was seen. On the test day, the saline-treated group of mice showed a significant preference for the cherry flavor due to two mice preferring the cherry flavor over the grape. However, when a metaanalysis was performed, the flavor preference did not affect other individual animals' preference for flavor in the other treatment groups.

Statistics

Statistical analyses were performed using PRISM (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± SEM. For Figure 1, a repeated measures two-way ANOVA was used to determine the effect of time on drug treatment followed by an unpaired Student's (two-tailed) t test to determine significance at each time point. Significance was determined using an unpaired Student's (two-tailed) t test Figure 2, and

Figure 5. For Figure 2B, a repeated measures two-way ANOVA was used to assay the effects of time across the different treatment paradigms (surgery and drug) followed by one-way ANOVA with Bonferroni's tests posthoc to determine significance across individual treatments. One-way ANOVA with Dunnett's test posthoc was used to determine significance across treatments in Figure 3, and Figure 5. Significance was taken as $p < 0.05$.

RESULTS

Intact leptin signaling is not essential for the actions of PYY_{3-36}

Signals relaying information about long-term energy stores and immediately available energy are integrated at key sites in the brain (for review, see (Wang et al., 2000)) or (Ellacott and Cone, 2004)). It has been shown previously, for example, that leptin and CCK can act synergistically to inhibit food intake (Barrachina et al., 1997; Emond et al., 1999b; Matson et al., 1997). Furthermore, rodents with deficient leptin signaling are hyposensitive to peripherally administered CCK (McLaughlin and Baile, 1980; Niederau et al., 1997). To test the hypothesis that the anorexigenic effects of PYY_{3-36} might be abolished in the absence of leptin signaling, we examined the ability of PYY_{3-36} to reduce food intake in Lep^{ob}/Lep^{ob} and $Lepr^{db}/Lepr^{db}$ mice, two models of obesity caused by a disruption in the ligand or the receptor, respectively, required for leptin signaling. In a nocturnal free-feeding paradigm, PYY_{3-36} (5 μg/100 g) was injected ip in Lep^{ob}/Lep^{ob}

and/or $Lepr^{db}/Lepr^{db}$ mice right before lights out (1900 h), and their food intake was measured every 2 h for 6 h. $PYY_{3–36}$ transiently but significantly reduced food intake in Lep^{ob}/Lep^{ob} mice in the first 4 h (Figure 1A). Lep^{ob}/Lep^{ob} mice treated with the peptide ate 47% ± 11% less food in the first 2 h and 39% ± 15% less food in 4 h compared to saline-treated controls (Figure 1A), levels nearly identical to the reduction seen in wt mice by the same dose of $PYY_{3–36}$ (Figure 2B). By 6 h, there was no difference in food consumption between saline and $PYY_{3–36}$-treated Lep^{ob}/Lep^{ob} mice (Figure 1A). Likewise, ip $PYY_{3–36}$ significantly reduced food intake in $Lepr^{db}/Lepr^{db}$ mice for the same duration and to the same degree (Figure 1B). The peptide treated group of $Lepr^{db}/Lepr^{db}$ mice ate 46% ± 9% less food at 2 h and 38% ± 15% less at 4 h compared to saline-treated controls (Figure 1B), also a nearly identical reduction in food intake to wt mice by the same dose of $PYY_{3–36}$ (Figure 2B). As in Lep^{ob}/Lep^{ob} mice, the effect of $PYY_{3–36}$ in $Lepr^{db}/Lepr^{db}$ mice was transient; no difference in food consumption was observed at 6 h (Figure 1B).

The vagus nerve is not essential for the inhibition of feeding by $PYY_{3–36}$

Gut peptides implicated in the acute regulation of hunger and satiety, such as ghrelin and CCK, have been shown to require the vagus nerve for their actions (Date et al., 2002; Smith et al., 1985). We postulated that $PYY_{3–36}$, a postprandially released gut peptide, may also require the vagus nerve to mediate its anorexic activity. To test this hypothesis, we injected $PYY_{3–36}$ or saline ip in wt mice with both vagus nerves surgically transected below the level of the diaphragm (BSDV mice) and compared their responses to animals

whose vagus nerves were surgically isolated and separated but not transected (Sham mice). We chose a nocturnal free-feeding paradigm to assay the effects of PYY_{3-36} on food intake because we found it eliminated the reduction of food intake in vagotomized mice that occurs due to stress in a fast-induced refeeding paradigm. Using CCK-8, we demonstrated that both vagus nerves were completely transected in BSDV mice and that Sham mice did not have their vagus nerves accidentally cut (Figure 2A). CCK-8 (10 μg/kg ip) significantly reduced 2 h nocturnal food intake in the Sham surgical group but not in the BSDV group (Figure 2A).

We also tested the ability of PYY_{3-36} (5 μg/100 g) injected ip immediately before lights out (1900 h) to reduce food intake in Sham and BSDV mice by measuring cumulative food intake every 2 h for 8 h. PYY_{3-36} reduced food intake to the same extent, 54% ± 7% in Sham and 46% ± 13% in BSDV mice in the first 2 h, as compared to saline-matched controls for each surgical group (Figure 2B). However, PYY_{3-36} showed a significantly different effect across time with different surgical treatments (Sham versus BSDV), as assayed by a repeated measures two-way ANOVA. In the Sham group, at 4 h, PYY_{3-36} significantly reduced food intake by 25% ± 10%, and only a trend toward reduction of food intake, without significance, was observed by 6 h (Figure 2B). In contrast, the BSDV group responded to PYY_{3-36} for an extended period of time, such that even by 6 h, PYY_{3-36} treated animals ate ~30% less than saline-treated BSDV and PYY_{3-36}-treated Sham mice (Figure 2B). In fact, at 8 h post injection, a significant reduction of food intake was measured in the PYY_{3-36}-treated BSDV mice as compared to saline-treated

Sham controls (Figure 2B), suggesting that BSDV mice actually exhibit prolonged sensitivity to the anorexigenic actions of a single bolus dose of $PYY_{3–36}$.

Dose-dependent activation of AP and intermediate NTS neurons with nocturnal peripheral administration of $PYY_{3–36}$

In order to begin to address the mechanisms by which $PYY_{3–36}$ inhibits food intake, it was important to characterize the minimum dose of peripheral $PYY_{3–36}$ required to inhibit food intake reliably and reproducibly. We previously showed that $PYY_{3–36}$ dose-dependently and transiently reduces food intake at high doses (3 and 10 μg/100 g) both in fasted and in nocturnal free-feeding paradigms in acclimated mice (Halatchev et al., 2004). However, at the lowest dose, 0.3 μg/100 g, $PYY_{3–36}$ neither reliably nor significantly reduced food intake at any time point in either feeding paradigm. Here, using a 16 h fast-induced refeeding paradigm, acclimated wt mice were injected ip with increasing doses of $PYY_{3–36}$ (0.3, 1.5, and 5 μg/100 g), and their cumulative food intake was measured hourly for 4 h. $PYY_{3–36}$ dose-dependently reduced food intake for the duration of the experiment (Figure 3A). As shown previously, although a trend for reduction in food intake at 0.3 μg/100 g of $PYY_{3–36}$ was observed, statistical significance was not reached at that dose compared to saline treatment (Figure 3A). However, $PYY_{3–36}$ significantly and reliably reduced food intake at the 1.5 μg/100 g dose (Figure 3A) at 1, 2, and 3 h. The highest dose of $PYY_{3–36}$ (5 μg/100 g) tested significantly reduced food intake for the duration of the experiment, 4 h (Figure 3A), as it did in the nocturnal

feeding paradigms in Sham, BSDV (Figure 2B), *Lep^ob^/Lep^ob^* (Figure 1A), and *Lepr^db^/Lepr^db^* mice (Figure 1B).

We next wanted to identify specific sites in the brain that are activated by peripherally administered $PYY_{3–36}$. We decided to focus on two brainstem nuclei in particular, the AP and NTS, both known to be involved in mediating the effects of gut-released peptides like CCK and ghrelin (Date et al., 2002; Fan et al., 2004). Nocturnal ip injection (1900 h) of increasing doses of $PYY_{3–36}$ (0.3, 1.5, and 5 μg/100 g) caused a significant dose-dependent activation of c-Fos IR in neurons in AP and intermediate NTS (Figure 3C, 3F, 3I, 3K, and 3L), compared to saline injections (Figure 3B, 3E, 3H, 3K, and 3L). The minimum peripheral dose of $PYY_{3–36}$ to reliably and reproducibly inhibit food intake, 1.5 μg/100 g (Figure 3A), was also the minimal dose to show a 2-fold statistically significant activation of c-Fos in the intermediate NTS, compared to a saline ip injection (Figure 3K). While increasing doses of ip $PYY_{3–36}$ consistently induced c-Fos IR in a very discrete subnucleus of the NTS, the positive control, CCK-8 (10 μg/kg), dramatically induced widespread c-Fos expression in neurons throughout the entire NTS and AP (Figure 3D, 3G, 3J, 3K, and 3L) to levels much greater than even the highest dose of $PYY_{3–36}$ (Figure 3C, 3F, 3I, 3K, and 3L).

Peripheral administration of $PYY_{3–36}$ causes conditioned taste aversion in mice

A growing number of reports and observations have failed to identify neuronal and/or hormonal systems required for the anorexigenic actions of $PYY_{3–36}$. However, previous

reports have demonstrated a link between PYY_{1-36} and emesis in dogs (Harding and McDonald, 1989; Perry et al., 1994). Additionally, in this report, we have shown that there is a dose-dependent, statistically significant activation of c-Fos IR, after peripheral administration of PYY_{3-36}, in intermediate NTS and AP, nuclei known to mediate the response to aversive stimuli (Schafe et al., 1995). This led us to the hypothesis that peripherally administered PYY_{3-36} may be mediating its anorexic effects through visceral illness. We chose a two-bottle, two-flavor conditioned taste aversion assay (Figure 4) to test whether PYY_{3-36}, at doses that reliably and reproducibly reduce food intake (1.5 and 5 μg/100 g), causes conditioned taste aversion in mice. Both peripheral doses of PYY_{3-36} (1.5 and 5 μg/100 g) significantly reduced drinking of the flavor associated with peptide administration (PYY_{3-36}-paired flavor) compared to the flavor associated with the saline treatment (saline-paired flavor) (Figure 5A). Saline-treated animals (saline pairing to both flavors) showed no preference for either bottle (Figure 5A). Intriguingly, the minimum peripheral dose of PYY_{3-36} to reliably and reproducibly inhibit food intake, 1.5 μg/100 g (Figure 3A), and significantly activate c-Fos IR in intermediate NTS (Figure 3K) also caused a significant reduction in consumption of the PYY_{3-36}-paired flavor, compared to the saline-paired flavor (Figure 5A). Mice that received 1.5 μg/100 g PYY_{3-36} drank 31% ± 17%, and mice that received 5 μg/100 g PYY_{3-36} drank 50% ± 15% less of the PYY_{3-36}-paired versus the saline-paired flavor (Figure 5A) at 1 h. The largest reduction of stimulus-paired flavor consumption was seen in the LiCl group (positive control), in which the difference between LiCl-paired and saline-paired flavor consumption was 96% ± 2% (Figure 5A) at 1 h.

To directly compare the effect of the treatment (saline versus different peptide concentrations and LiCl) on the relative consumption of the stimulus-paired flavor, we also expressed the results in ratios, in which the amount of stimulus-paired flavor is divided by the total volume (see Experimental Procedures) consumed by an animal (Figure 5B). PYY_{3-36} dose-dependently and significantly reduced the ratios, compared to saline control (Figure 5B). Compared to the saline control ratio of 0.52 ± 0.05 ($n = 17$), PYY_{3-36} had a ratio of 0.42 ± 0.05 (ns; $n = 17$) at 1.5 µg/100 g PYY_{3-36} dose and 0.33 ± 0.06 ($p < 0.0115$; $n = 17$) at 5 µg/100 g PYY_{3-36} dose (Figure 5B). The drastic reduction of stimulus-paired flavor consumed by the LiCl-treated group translated to a ratio of 0.05 ± 0.03 ($p < 0.0001$; $n = 9$) (Figure 5B).

DISCUSSION

Two decades after the original discovery of PYY by Tatemoto in 1980, it was demonstrated that peripheral administration of the Y2 receptor-preferring PYY_{3-36} form of the peptide reduced food intake in mice, rats, and humans (Batterham et al., 2002). In their study, Batterham et al., 2002 also reported that PYY_{3-36} decreases the tonic inhibitory drive from NPY neurons onto POMC neurons in the ARC by activating inhibitory Y2 autoreceptors on NPY neurons or terminals. Furthermore, they demonstrated that peripheral administration of PYY_{3-36} caused activation of a small percentage (~12%) of POMC neurons in ARC, assessed by expression of c-Fos, a marker of neuronal activation. From these observations, the effects of PYY_{3-36} on food intake

were proposed to be mediated through the central melanocortin system. However, since this report, a number of published observations have raised serious doubts about the role of the central melanocortin system in the anorexigenic effects of peripherally administered PYY_{3-36}. Mice lacking the POMC gene (Challis et al., 2004), the melanocortin-4 receptors (Halatchev et al., 2004) or melanocortin-3 receptors (Appendix Fig. 2) are fully responsive to the peptide. Likewise, lean and obese agouti mice are also fully responsive to peripheral injection of PYY_{3-36} (Martin et al., 2004). Nevertheless, PYY_{3-36} is still considered by some to be a potentially promising therapeutic agent, since it appears to reduce food intake in lean and obese humans (Batterham et al., 2003; Batterham et al., 2002) as well as in normal and obese mice (Pittner et al., 2004). In two studies from the same laboratory, human subjects were infused with the peptide intravenously and showed 33% reduction of calories consumed in a meal. Additional studies will be necessary to validate these observations in the human as well as to determine if there is any clinical efficacy in the treatment of obesity. Nevertheless, elucidation of mechanisms by which this postprandially-released gut peptide reduces food intake remains highly relevant.

A variety of peripheral peptides that relay information about energy availability have synergistic effects on key circuits in the brain involved in regulation of energy intake and expenditure. For example, leptin, the adipostatic hormone released from adipose tissue, has synergistic actions with CCK (Barrachina et al., 1997; Emond et al., 1999b; Matson et al., 1997). However, animal models lacking the key signal relaying long-term energy availability, like rodents lacking leptin signaling, show desensitized responses to bolus

injections of some satiety factors. CCK, for example, shows a decreased ability to induce satiety in a number of obese rodent models, like MC4-$R^{-/-}$ mice and obese Zucker rats (Halatchev et al., 2004; McLaughlin and Baile, 1980; Niederau et al., 1997). In contrast, bolus peripheral injections of PYY_{3-36} reduce food intake to the same amount and time duration in MC4-$R^{-/-}$ (Halatchev et al., 2004), MC4-$R^{-/-}$ (Appendix Fig. 2), Agouti (Martin et al., 2004), and $POMC^{-/-}$ mice (Challis et al., 2004) as they do in wt mice. Therefore, we wanted to test the efficacy of PYY_{3-36} to reduce food intake in mice lacking the leptin gene Lep^{ob}/Lep^{ob} and in mice with a mutation in the long form of the leptin receptor $Lepr^{db}/Lepr^{db}$. PYY_{3-36} reduced food intake, using a nocturnal feeding paradigm, in Lep^{ob}/Lep^{ob} and $Lepr^{db}/Lepr^{db}$ mice (Figure 1). This experiment shows that intact leptin signaling is not essential for the anorexigenic actions of PYY_{3-36}. Furthermore, the lack of any potent desensitization to PYY_{3-36} in animals lacking leptin signaling argues that PYY_{3-36}, in contrast to CCK, may not act via a leptin-regulated satiety pathway.

We next wanted to determine if the effects of PYY_{3-36} on food reduction require an intact vagus nerves, like the gut-derived factors CCK and ghrelin (Date et al., 2002; Smith et al., 1985). We chose a nocturnal nonfasted feeding paradigm to assay the effects of peripherally administered PYY_{3-36} on vagotomized mice because we and others have observed that fasted vagotomized animals consume less food than sham-operated controls, presumably due to stress or gastric distention (Moran et al., 1997; Reidelberger, 1992). Our previous observation demonstrated that stress could easily mask the anorexigenic effects of PYY_{3-36} (Halatchev et al., 2004).

PYY_{3-36} (5 μg/100 g) reduced food intake to the same extent in vagotomized mice and Sham controls (Figure 2B). However, in contrast to Sham mice, PYY_{3-36} had a prolonged duration of effect in vagotomized mice at that dose, reducing food intake for up to 8 h (Figure 2B). This observation was quite striking; instead of attenuating the effects of PYY_{3-36} on food intake, total (afferent and efferent) subdiaphragmatic vagotomy, in fact, prolonged them. This may reflect that even though an intact vagus nerve is not required for the short-term anorexigenic actions of peripheral PYY_{3-36}, unlike CCK and ghrelin, intact vagal tone could modulate the duration of action of PYY_{3-36}. Moreover, it has been shown in the literature that intact efferent vagal parasympathetic tone modulates basal and food-induced release of PYY (Zhang et al., 1993b), as well as some of the ability of the peptide to modulate gastrointestinal (GI) transit time and glandular secretion (Chen et al., 1996; Masuda et al., 1994). In contrast to the results reported here, a recent study has demonstrated blockade of PYY_{3-36}-mediated inhibition of food intake in the rat by bilateral subdiaphragmatic vagotomy (BSDV) (Koda et al., 2005). Additional work will be required to determine if the different findings result from species differences between rat and mouse or from methodological differences. For example, regulation of growth hormone release by ghrelin appears to be dependent on the vagus in rats (Date et al., 2002) but not in humans (Takeno et al., 2004).

Even though dose response curves for PYY_{3-36} can be found in the literature showing the magnitude and duration of food reduction by PYY_{3-36}, the minimum dose required to produce significant and reliable food inhibition by ip administration has not been reported

in mice. Here we show that the minimum dose of PYY_{3-36} needed to reliably and reproducibly cause a short-term inhibition of food in mice is 1.5 μg/100 g (Figure 3A), with varying duration of action from 1 to 3 h (data not shown). This dose was then used in both the neuroanatomical and behavioral tests performed in the rest of this study.

Certain sites in the brain have been implicated in mediating the effects of gut peptides and other hormones in the regulation of satiety and long-term energy homeostasis. The hypothalamus has been proposed to be a primary site of action of the long-term adipostatic factor leptin (Elias et al., 1999). In the brainstem, the NTS is the primary site for reception of satiety signals from the vagus, provided by both neural and hormonal signals from the gut, such as gastric distension and CCK (Jean, 1991). Other gut-derived factors, like amylin, appear to be vagus independent and act directly on brainstem structures like the AP to inhibit feeding (Lutz et al., 1995; Lutz et al., 2001; Riediger et al., 2002). It was thus, perhaps, surprising that a gut-released peptide, like PYY_{3-36}, would mediate its short-term anorexigenic effects directly via the ARC of the hypothalamus. Additional work will be required to determine the mechanism and relevance of activation of small numbers of arcuate POMC neurons by PYY_{3-36}. However, in this report, we looked at the ability of peripherally administered PYY_{3-36} to activate neurons throughout the entire NTS and other brainstem nuclei known to be part of the gut-brain axis. Specifically, we focused on the AP, a circumventricular organ, and the NTS, two sites known to be involved in satiety and emesis. In a previous report, focusing on a different region of the NTS expressing POMC, we did not observe activation of neurons in the NTS by PYY_{3-36} (Halatchev et al., 2004). However, here,

after examining the entire NTS, we observed a dose-dependent activation of AP and NTS neurons with increasing concentrations of PYY_{3-36}, as assayed by c-Fos IR, a marker of neuronal activation (Figure 3). In contrast to CCK, which dramatically activated neurons throughout the AP and NTS, PYY_{3-36} activated a specific subnucleus of the NTS, the intermediate nucleus of the NTS (Figure 3). Even though both peptides, CCK and PYY_{3-36}, are secreted in response to fatty acids in the GI lumen and reduce food intake by similar amounts and over similar time courses, the strikingly different pattern of c-Fos activation in NTS and AP likely reflects inherent differences in their mechanisms of action. A functional example of this is the fact that CCK requires MC4-R signaling for the inhibition of food intake while PYY_{3-36} does not (Fan et al., 2004; Halatchev et al., 2004).

Aversive stimuli are well established to activate specific neuronal populations within AP and intermediate NTS, such as those induced by ip injection of LiCl (Schafe et al., 1995). In fact, we observed a very similar pattern of activation of c-Fos in the same regions of the intermediate NTS, as observed immediately after ip LiCl and/or in response to a flavor that has been associated with an aversive stimulus (Houpt et al., 1997; Schafe et al., 1995), such as LiCl. Furthermore, Schafe et al., 1995 have demonstrated that the acquisition of the aversive memory to a substance, required for conditioned taste aversion, seems to be dependent on an intact forebrain-hindbrain relay through nerve tracts above the superior colliculus (Schafe et al., 1995). Here we show that a single bolus ip injection of PYY_{3-36} produces c-Fos IR in the same brainstem nuclei as LiCl and reduces food intake. Thus, it is consistent with these findings that, like LiCl, PYY_{3-36}

may, at least in part, reduce food intake by causing a short-lived aversive response. Additional data will be required, however, to determine if PYY_{3-36} treatment activates the same subgroup of intermediate NTS neurons as other aversive substances like LiCl.

A number of gut-released peptides have been shown to mediate part of their effects on food intake through induction of an aversive response. At high doses, CCK causes taste aversion and activates neurons in AP and NTS (Deutsch and Hardy, 1977; Ervin et al., 1995). However, unlike other well-characterized substances that cause an aversive response, like LiCl, the association produced by high doses of peripheral CCK is dependent on the vagus nerve (Verbalis et al., 1986). Here we wanted to examine whether the dose-dependent inhibition of food intake and c-Fos activation by PYY_{3-36}, in the AP and intermediate NTS, might be due to the production of a short-term aversive response in mice. At doses that reliably and reproducibly inhibit short-term food intake, PYY_{3-36} caused conditioned taste aversion to a flavor paired with PYY_{3-36} treatment (Figure 5). These data raise the possibility that the primary mechanism by which PYY_{3-36} inhibits food intake in mice is through a short-term aversive response.

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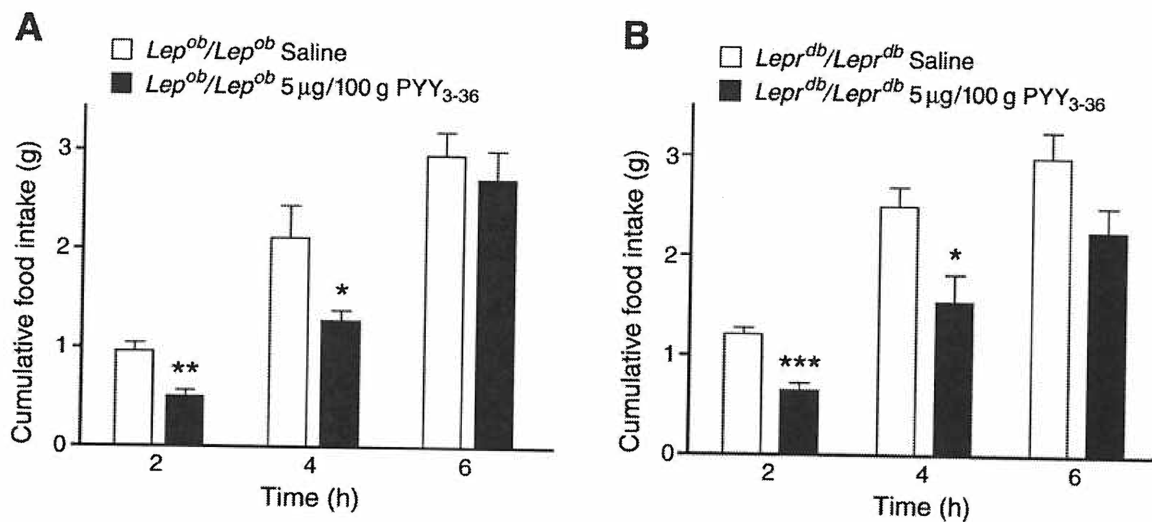


Fig. 1. *Lep*^{ob}/*Lep*^{ob} and *Lepr*^{db}/*Lepr*^{db} mice respond equivalently to peripherally administered $PYY_{3\text{–}36}$ in a nocturnal feeding paradigm. **A,** Nocturnal feeding response of *Lep*^{ob}/*Lep*^{ob} mice to saline or a $PYY_{3\text{–}36}$ dose of 5 µg/100 g at 2, 4, and 6 hr post injection (n = 6, each group). **B,** Nocturnal feeding response of *Lepr*^{db}/*Lepr*^{db} mice to saline or a $PYY_{3\text{–}36}$ dose of 5 µg/100 g at 2, 4, and 6 hr post injection (n = 5, each group). Data are expressed as mean ± SEM, statistics by repeated measures two-way ANOVA followed by a Student's (two-tailed) t test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

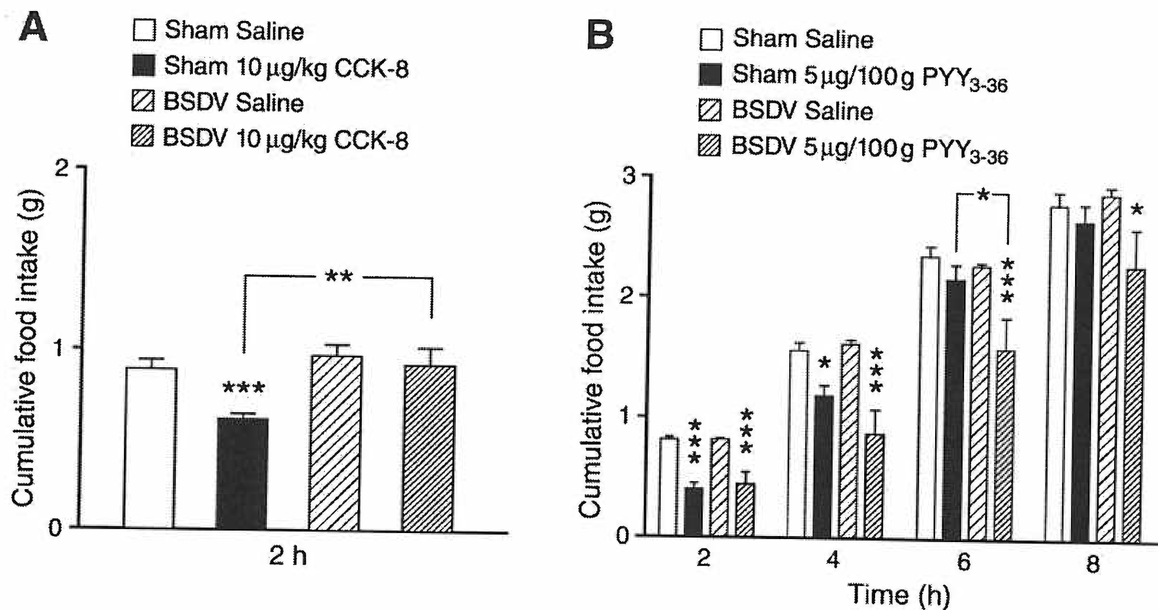
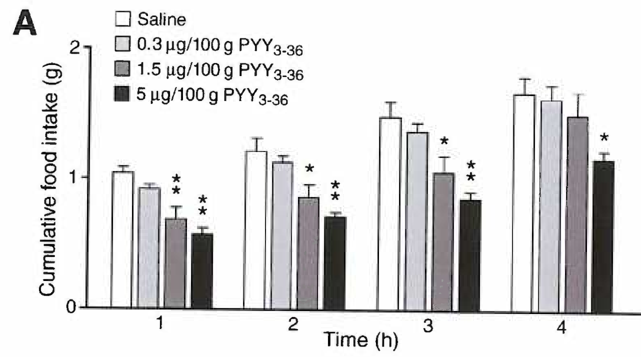
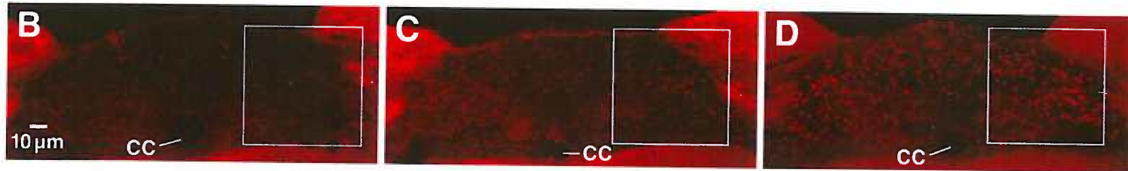
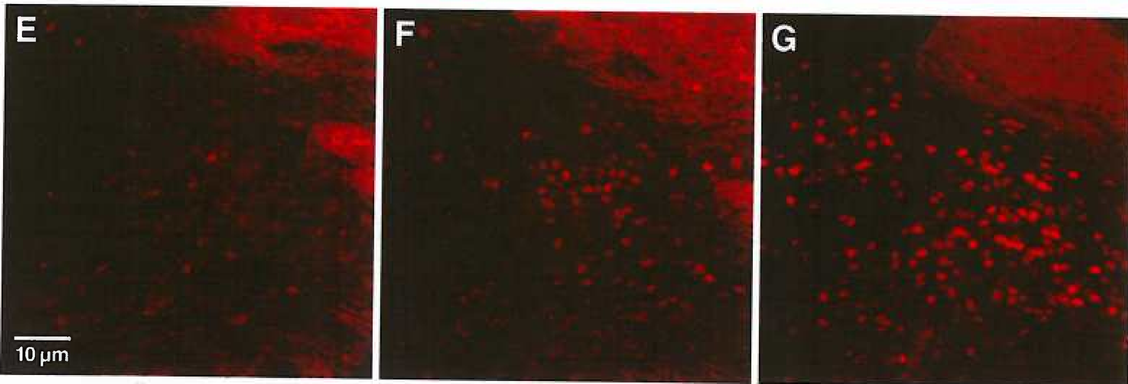


Fig. 2. Intact vagus nerves are not essential for the actions of peripherally administered PYY_{3-36} in a nocturnal feeding paradigm. **A,** Nocturnal feeding responses of Sham and BSDV mice to i.p. CCK-8 dose of 10 µg/kg at 2 hr post injection (Sham saline and CCK-8, n = 10; BSDV saline and CCK-8, n = 6). **B,** Nocturnal feeding responses of Sham and BSDV mice to i.p. PYY_{3-36} dose of 5 µg/100g at 2, 4, 6, and 8 hr post injection (Sham saline and CCK-8, n = 10; BSDV saline and CCK-8, n = 6). Data are expressed as mean ± SEM, statistics by Student's (two-tailed) t test for (**A**) and repeated measures two-way ANOVA followed by one-way ANOVA with Bonferroni's tests posthoc for each time point in (**B**), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.


A
Saline
0.3 µg/100 g PYY3-36
1.5 µg/100 g PYY3-36
5 µg/100 g PYY3-36
Cumulative food intake (g)
Time (h)


B
C
D
10 µm
CC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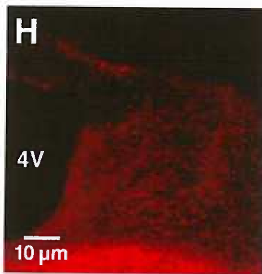
H
4V
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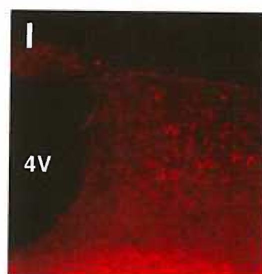
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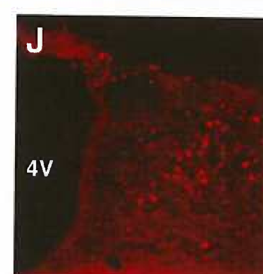
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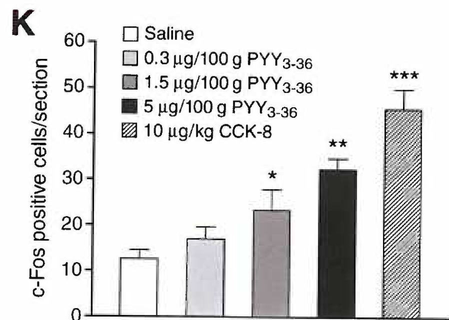
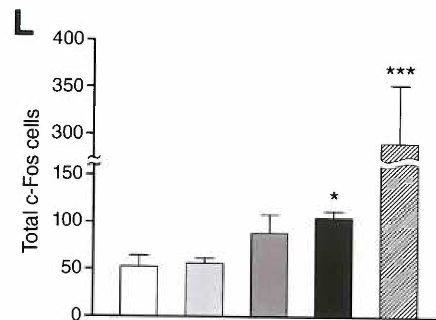

K
Saline
0.3 µg/100 g PYY3-36
1.5 µg/100 g PYY3-36
5 µg/100 g PYY3-36
10 µg/kg CCK-8
c-Fos positive cells/section


L
Total c-Fos cells

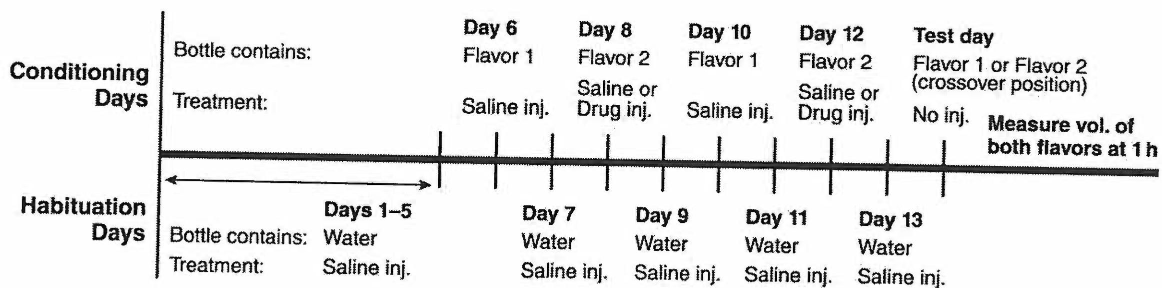


Fig. 4. Schematic timeline of the conditioned taste aversion protocol. All male age-matched mice were individually housed and received 1 hr of water or flavor in two separate bottles (equidistant from the food) daily for 13 days. Each animal was weighed and i.p. injected daily with either saline (habituation days, days 1–7, 9–11, and 13, or saline/peptide/LiCl days, 8 and 12). On the test day, mice received both flavors in a crossover position, and intake from each bottle was measured after 1 hr.

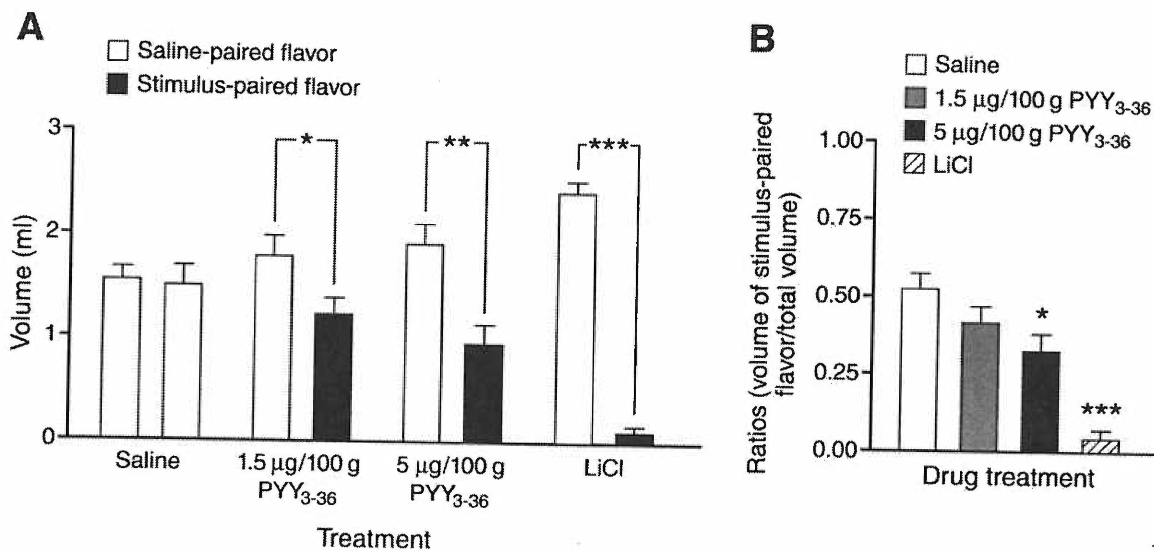


Fig. 5. Peripheral administration of PYY_{3-36} at doses that reliably inhibit food intake causes conditioned taste aversion in wt mice. **A,** Individual flavor consumption (saline-paired flavor versus stimulus-paired flavor) after stimulus-paired flavor is associated with i.p. LiCl (2% BW of 0.15 M LiCl) or PYY_{3-36} (1.5 and 5 µg/100 g) treatment. **B,** Ratios of volume of stimulus-paired flavor consumed versus total volume consumed across treatment groups (saline, 1.5 and 5 µg/100 g, n = 17; LiCl, n = 9). Data are expressed as mean ± SEM, statistics by Student's (two-tailed) t test for (**A**) and one-way ANOVA with Dunnett's test post-hoc for (**B**), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

CHAPTER THREE

Inhibition of food intake by PYY_{3-36} in NPY/AgRP knockout mice and monosodium glutamate treated animals

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ABSTRACT

Peptide YY_{3-36} (PYY_{3-36}) is a postprandially released gut peptide, demonstrated to inhibit food intake. Initial reports suggested that effects of PYY_{3-36} on food intake are mediated by the central melanocortin circuitry in the arcuate nucleus (ARC). In initial reports, bath applied PYY_{3-36} was observed to cause presynaptic inhibition of arcuate neuropeptide tyrosine (NPY) neurons and the subsequent activation of arcuate proopiomelanocortin (POMC) neurons, generating a net anorexigenic drive to reduce food intake. However, more recent data have demonstrated that the anorexigenic activity of PYY_{3-36} is not dependent on melanocortin ligands or their receptors in the CNS. We demonstrate here that the anorexigenic actions of PYY_{3-36} are independent of NPY and AgRP signaling. We further examined the ability of peripheral PYY_{3-36} to reduce food intake and induce c-Fos, a marker of neuronal activation, in mice with their ARC nuclei lesioned by postnatal MSG treatment. Peripherally administered PYY_{3-36} significantly reduced food intake in monosodium glutamate (MSG)-treated mice. Furthermore, MSG-treatment abolished the c-Fos induced by peripheral PYY_{3-36} in the ARC, while sparing the activation of the intermediate nucleus tractus solitarius (iNTS) of the brainstem. Thus, the inhibition of food intake by PYY_{3-36} appears to be partially mediated through the brainstem and does not require the presence of an intact ARC.

INTRODUCTION

PYY_{3-36}, a secreted gut peptide, has been shown to inhibit food intake in rats and mice (Batterham et al., 2002). This peptide has achieved additional attention due to the observation that continuous intravenous infusion of PYY_{3-36} reduced 24 h food intake and hunger scores in a small number of lean and obese human subjects, suggesting a potential clinical utility of the peptide in the treatment of obesity (Batterham et al., 2003; Batterham et al., 2002).

PYY_{3-36} is one of two isoforms of peptide tyrosine tyrosine (PYY). PYY_{1-36} is a member of the pancreatic polypeptide (PP) family, which also includes NPY and PP, and mediates its biological activity through the NPY family of receptors (Y1-5) (for review see (Keire et al., 2002)).

PYY_{1-36} is secreted from L-cells in the distal small intestine and colon in response to ingested nutrients (Adrian et al., 1985; Bottcher et al., 1986; Bottcher et al., 1993). In the circulation, dipeptidyl peptidase-IV (DPP-IV) cleaves approximately 40% of PYY_{1-36} to produce PYY_{3-36} (Grandt et al., 1994b). This cleavage confers specificity of PYY_{3-36} predominantly for the Y2 (Ki=0.03-0.3 nM) autoinhibitory NPY receptor (Keire et al., 2002).

The nucleus tractus solitarius (NTS) POMC and NPY neurons are well positioned to respond to short-term energy availability and satiety. Recently, the satiating effects of cholecystokinin (CCK), the archetypal satiety peptide released postprandially from the

gut, were shown to be dependent on the brainstem melanocortin system (Fan et al., 2004). The proposed mechanism by which CCK is thought to cause satiety, under physiological conditions, is as follows: CCK is released from the gut in response to ingested nutrients and acts through CCK-A receptors on the vagus (Ritter and Ladenheim, 1985) broadly activating a variety of NTS neurons (Fan et al., 2004), which produces an anorexigenic drive, terminating the meal.

The inhibition of feeding, observed after peripheral administration of PYY_{3-36}, was initially thought to be due to the generation of a net anorexigenic drive by the ARC NPY/AgRP and POMC neurons (Batterham et al., 2002). In this model, circulating PYY_{3-36} could gain direct access to the ARC melanocortin circuitry, either through the median eminence (ME) or via the blood-brain barrier (BBB). Using an in vitro ARC slice preparation, bath applied PYY_{3-36} was observed to reduce the inhibitory postsynaptic potentials onto POMC neurons, presumably binding presynaptic Y2 autoinhibitory receptors present on NPY terminals thus inhibiting the tonic inhibitory drive of NPY neurons (Batterham et al., 2002). Additionally, peripherally administered PYY_{3-36} significantly induced c-Fos, a marker of neuronal activation, across the ARC nucleus and within ARC POMC neurons (Batterham et al., 2002; Halatchev et al., 2004).

However, numerous recent reports have brought some aspects of this model into question. Firstly, the anorexigenic effects of PYY_{3-36} are not dependent on intact melanocortin agonist signaling since peripherally administered PYY_{3-36} inhibits food intake in, MC4-R knockout ($MC4\text{-}R^{-/-}$) (Halatchev et al., 2004), Agouti (Martin et al.,

2004), and POMC knockout mice ($POMC^{-/-}$) (Challis et al., 2004) equally to wild-type (wt), sex and age matched controls. Secondly, two reports have raised the possibility that the ARC nucleus may not be the primary site of action of peripheral PYY_{3-36}. In one, peripheral administration of PYY_{3-36} was shown to cause taste aversion in mice and a dose dependent activation of c-Fos in AP and intermediate NTS (iNTS) (Halatchev and Cone, 2005). The iNTS and AP are areas shown to respond to aversion (Schafe et al., 1995). Second, in the rat, vagotomy abolishes the induction of ARC c-Fos by peripherally administered PYY_{3-36} (Koda et al., 2005).

In this study, we sought to determine if the anorexigenic effect of PYY_{3-36} involves blockade of orexigenic stimulus from the AgRP and NPY signaling, or more generally requires a functionally intact ARC nucleus. We first tested if NPY/AgRP double knockout mice are responsive to the anorexigenic actions of PYY_{3-36}. We also examined the ability of peripherally administered PYY_{3-36} to reduce food intake in mice with their ARC nuclei ablated by postnatal treatment with monosodium glutamate (MSG). Finally, we examined the ability of peripherally administered PYY_{3-36} to induce c-Fos activation in ARC and NTS of MSG-treated mice.

MATERIALS AND METHODS

Animals

Hemizygous NPY-Sapphire transgenic mice, expressing the Tau-Sapphire (green fluorescent protein variant) fusion protein driven under NPY genomic elements, were derived from animals described previously (Pinto et al., 2004). NPY-Sapphire mice were a kind gift of Dr. Hongyan Liu and Dr. Jeffrey Friedman of Rockefeller University (New York, NY) and were received on a mostly C57Bl/6J background with a smaller CBA contribution (Pinto, S., personal communication). NPY/AgRP double knockout mice were generated by crossing NPY knockout ($NPY^{-/-}$) mice with AgRP knockout mice ($AgRP^{-/-}$) mice in a 129SV/C57BL/6J hybrid background, as described previously (Qian et al., 2002). All transgenic animals were raised in group housing with their siblings and maintained at 23°C ± 1°C on a 12 h light, 12 h dark cycle (0700–1900 h light). Mice were allowed ad libitum access to standard chow pellets (Purina Laboratory Rodent Diet 5001, Ralston Purina Co., St. Louis, MO; ~4.5% fat by weight). WT experimental animals of the C57BL/6J strain were purchased to be age, sex, and weight matched (The Jackson Laboratory, Bar Harbor, ME). Upon arrival wt mice were allowed to acclimate for 1 week under the conditions stated above. All studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the animal care and use committee of Oregon Health and Science University.

Source of reagents/peptides

All experiments were performed with human $PYY_{3\text{–}36}$ (Bachem, Torrance, CA; lot 0562660). Peptides were certified by the manufacturer and came with HPLC data showing a single peak with the correct molecular weight from mass spectrogram and a purity grater than 97%. Peptides were dissolved in sterile isotonic saline and injected in a total volume of 500 μl/injection. Fresh human $PYY_{3\text{–}36}$ was prepared on the day of the injection from frozen stock solutions.

Postnatal monosodium glutamate treatments (MSG)

Pups from heterozygous breeding pairs of NPY-sapphire mice bred to WT were injected subcutaneously (sc) on postnatal day 2, 4, 6, 8, and 10 with 4 mg/g body weight (BW) with MSG. Age matched pups from WT breeding pairs were injected with saline on the same postnatal days. Over 95% of pups survived the treatment paradigm, independent of injection type. Mice, both saline-treated and MSG-treated, were weaned 3 weeks of age and placed on soft food (standard chow pellets in water) for 2 weeks because MSG-treated mice were ~50% smaller than their saline-treated controls. Female saline-treated and MSG-treated animals underwent feeding experiments at 8 weeks of age, while male mice were 12 weeks old. Although MSG-treated mice remained ~50% shorter than their age and sex matched saline-treated controls, they became obese by 8 wk of age and matched the weight of their same sex saline-treated controls.

Feeding protocols

16 h fast induced refeeding in acclimated NPY/AgRP double knockout and WT mice

Age and sex matched NPY/AgRP double knockout and WT (mixed background controls) mice (8 weeks) were used for the feeding study as described previously (Halatchev et al., 2004). In short, animals were individually housed for a week. During the second week, animals were acclimated to the procedure by ip saline injections daily, with food in a petri dish and mock food measurements at 2 h and 4 h after injection. Mice were fasted for 16 h the night before the experiment (1900–1100 h). Animals were injected with either saline or PYY_{3-36} ip at a dose of 3, and 10 μg/100 g, and cumulative food intake was measured at 2 h and 4 h after injections by placing two pellets of chow in petri dishes on the floor of the cage. To minimize error attributable to loss of food particles or bedding in petri dishes, petri dishes and bedding were screened.

16 h fast induced refeeding in acclimated postnatally saline-treated and MSG-treated mice

Age and sex matched saline-treated and MSG-treated mice (8-12 weeks) were used for the feeding study as described previously (Halatchev et al., 2004), however, with minor modifications, due to the fact that MSG-treated mice are hypersensitive to stress and fasting (Dawson and Lorden, 1981). Mice were individually housed for a week. During the following two weeks, animals were acclimated to the procedure by ip saline injections daily, with food in petri dish and mock food measurements every hour for 4 h. After the two weeks long acclimatization to injections, mice were fasted every other day for 16 h (1900–1100 h) for four times, and injected with saline until their food intake stabilized. All mice were injected with saline on non-fasted days as well. On the morning after the fifth and the sixth fast, separated by 48 h, animals were injected with either saline or PYY_{3-36} ip at a dose 5 μg/100 g, in a cross-over manner, and cumulative food intake was

measured every hour for 4 h after injections by placing two pellets of chow in petri dishes on the floor of the cage. Measurement error was minimized with careful screening of petri dishes for debris and cage bedding for spilled food.

Labeling studies

General Fixing procedure

NPY-sapphire and wt mice were handled and injected with 500 μl sterile saline ip at 1900 h for 5 days prior to the experiment to minimize background c-Fos immunoreactivity caused by stress. During the acclimatization period, food was withdrawn from animals 2 h prior to injections (for 3.5 h) to minimize neuronal activation due to feeding (Fan et al., 2004). Animals received an ip injection of $PYY_{3\text{–}36}$ (5 μg/100 g) or sterile saline 90 min (at lights out, 1900 h) before anesthesia and transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). Sections from perfused brains (25 μM) were cut and stored free-floating in 0.01 M PBS containing 0.03% sodium azide.

Hematoxylin and eosin (H&E) staining

Sections were mounted on superfrost® plus slides (VWR Scientific, West Chester, PA). Sections were re-hydrated in two changes of absolute alcohol for 5 min each, followed by 2 min in 95% alcohol, 2 min in 70% alcohol and a brief wash with distilled water. Slides were dipped in Harris hematoxylin solution (100g potassium in 1L distilled water, heated to dissolve, 50 ml of 10% alcoholic hematoxylin solution added and brought to boil for 1 min, added 2.5g mercuric oxide, re-heated, cool in water bath and add 20ml glacoacetic

acid, filter) for 8 min, followed by 5 min wash in running water. After rinsing sections were differentiated in 1% acid alcohol (1 ml hydrochloric acid in 100ml 70% ethanol) for 30 sec, and re-washed for 1 min. Sections were treated with 0.2% ammonia water for 30 sec, re-washed for 5 min, followed by rinsing in 95% alcohol for 10 dips. Finally, sections were counter stained with eosin-phloxine solution (100ml eosin solution, 10ml phloxin solution, 780 ml 95% ethanol, and 4ml glacial acetic acid) for 30 sec and dehydrated by two changes in 95% alcohol for 5 min each. Slides were viewed with a light microscope (Axioplan 2; Zeiss Inc., Thornwood, NY). Pictures of representative ARC and NTS/AP sections were taken.

POMC immunohistochemistry

The sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS + 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-POMC precursor (Porcine) antibody (Phoenix Pharmaceuticals, Inc., Belmont, CA) and diluted 1:2,000 in blocking reagent for 48 h at 4°C, followed by incubation in 1:500 donkey anti-rabbit Alexa 594 (Molecular Probes Inc., Eugene, OR) for 1 h at room temperature. Between each stage, the sections were washed thoroughly with 0.01 M PBS. At the end of the incubations, the sections were mounted onto gelatin-coated slides, coverslipped using gel-based fluorescence mounting media (Biomeda Corp., Foster City, CA), and viewed under a fluorescence microscope (Axioplan 2; Zeiss Inc., Thornwood, NY). The number of POMC immunoreactive cells was counted in sections representing the ARC.

c-Fos immunohistochemistry

The sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS + 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-c-Fos (Ab-5), human (rabbit) polyclonal antibody (Oncogene Research Products, La Jolla, CA; Lot D21570) and diluted 1:70,000 in blocking reagent for 24 h at 4°C, followed by incubation in 1:500 donkey anti-rabbit Alexa 594 (Molecular Probes Inc., Eugene, OR) for 1 h at room temperature. Between each stage, the sections were washed thoroughly with 0.01 M PBS. At the end of the incubations, the sections were mounted onto gelatin-coated slides, coverslipped using gel-based fluorescence mounting media (Biomeda Corp., Foster City, CA), and viewed under a fluorescence microscope (Axioplan 2; Zeiss Inc., Thornwood, NY). The number of c-Fos immunoreactive cells was counted in sections of the ARC and the NTS.

Statistics

Statistical analyses were performed using PRISM (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± SEM. For Figure 1, and 4, a repeated measures two-way ANOVA was used to determine the effect of time on drug treatment followed by an unpaired Student's (two-tailed) t test to determine significance at each time point. Significance was determined using an unpaired Student's (two-tailed) t test Figure 5. Significance was taken as $p < 0.05$.

RESULTS

NPY/AgRP is not required for inhibition of feeding by PYY_{3-36}

Immunocytochemical studies have identified Y2 receptors on presynaptic terminals of the ARC NPY neurons (Broberger et al., 1997), and their activation by NPY leads to the inhibition of NPY release (Cowley et al., 2001b; King et al., 1999). PYY_{3-36} has very high specificity for the Y2 receptor with Ki=0.03-03 nM (for review see (Keire et al., 2002)). Indeed, bath applied PYY_{3-36} decreases the inhibitory postsynaptic potentials onto POMC neurons, presumably via Y2 receptors from NPY neurons (Batterham et al., 2002). Additionally, in hypothalamic explant studies, Y2 agonist reduced the release of NPY from nerve terminals (Batterham et al., 2002). Finally, hypothalamic NPY mRNA levels are reduced 6 h after peripheral administration with PYY_{3-36} (Challis et al., 2003). To test the hypothesis that the anorexigenic effects of ip PYY_{3-36} may result from reduction of orexigenic drive from the central NPY system, producing a net anorexigenic ARC drive, we examined the ability of PYY_{3-36} to reduce food intake in NPY/AgRP double knockout mice. Using a 16 h fast-induced refeeding paradigm, acclimated NPY/AgRP double knockout and wt littermate control mice were injected ip with 3 μg/100g or 10 μg/100g (Fig. 1) of PYY_{3-36}, and cumulative food intake was measured at 2 and 4 h. As shown previously with the C57BL/6J mouse strain, 129SV/C57BL/6J hybrid mice show a dose dependent inhibition of food intake with ip PYY_{3-36} (Fig. 1). PYY_{3-36} at the 3 μg/100g dose (Fig. 1A) non-significantly reduced food intake by 19 ±14% in the first 2 h, and by 8 ±14% at 4 h post injection, while at the 10 μg/100g dose

(Fig. 1B), PYY_{3-36} significantly reduced food intake by 26 ±13% and 24 ±10% at the same time points.

NPY/AgRP double knockout mice also exhibited a dose dependent significant decrease in food intake for the duration of the experiment, like wt controls, after ip injection of PYY_{3-36} (3 µg / 100 g and 10 µg / 100 g) (Fig. 1). At the 3 µg / 100 g dose of PYY_{3-36} food intake was reduced by 44 ±15% at 2 h and 39 ±15% at 4 h (Fig. 1A), while the 10 µg /100 g dose of PYY_{3-36} caused a reduction of 62 ±10% and 56 ±11%, respectively (Fig. 1B).

The ARC nucleus is not required for the anorexigenic actions of peripheral PYY_{3-36}

In addition to being expressed in NPY neurons (Broberger et al., 1997), Y2 receptor mRNA is also found, at high densities, in other key centers implicated in the regulation of energy homeostasis, like the NTS (Broberger et al., 1997; Parker and Herzog, 1999). Indeed, peripheral PYY_{3-36} could gain direct access to ARC and NTS through circumventricular organs, like ME and AP, closely associated with both nuclei, respectively. Initial observations showed that peripheral administration of PYY_{3-36} causes activation of c-Fos in approximately 12% of ARC POMC neurons (Batterham et al., 2002) and changes in the levels of ARC NPY and POMC mRNA (Challis et al., 2003). However, we recently showed that peripheral administration of PYY_{3-36} causes taste aversion and activates c-Fos in the AP and intermediate NTS in mice (Halatchev and Cone, 2005), two areas shown to be sufficient to mediate aversion and the satiating

effects of other gut hormones, like CCK (Fan et al., 2004). To determine whether the ARC nucleus is required for the anorexigenic actions of peripheral $PYY_{3–36}$ we evaluated the ability of MSG-treated mice to respond to ip $PYY_{3–36}$.

Postnatal MSG-treatment ablates the ARC nucleus of the hypothalamus, including the ARC NPY and POMC neuronal populations

The extent of the neurochemical lesion of the ARC produced with postnatal administration of MSG was analyzed by examining the brains of MSG-treated and saline-treated male littermates with either H&E stain, POMC immunocytochemistry, or NPY-Sapphire autofluorescence in NPY-Sapphire positive mice (Fig. 2). Comparison of H&E staining of ARC nucleus of postnatally MSG-treated vs. saline-treated mice (Fig. 2A and B) shows a marked hypocellularity in the MSG-treated mouse brains, exemplified by a large reduction of cell nuclei within the ARC. Furthermore, MSG-treatment produced a substantial dilation of the 3rd ventricle with almost complete loss of the ME (Fig. 2B). In NPY-Sapphire male mice which underwent MSG-treatment (n=4), no NPY neurons were detected in any of the ARC sections, compared to ~42 NPY-positive neurons per section observed in untreated NPY-Sapphire controls (Fig. 2 C, D, and E). However, the ablation of NPY-positive neurons was limited to the ARC, as NPY-positive neurons were observed in the cortex of MSG-treated mice (Fig. 2D insert). Immunocytochemistry for POMC revealed a marked reduction from ~85 to less than 4 POMC-positive neurons per section with MSG-treatment (Fig. 2F, G, and H). In association with the loss of NPY and POMC neuronal cell bodies, profound reduction of NPY and POMC fiber density was

observed, originating from the ARC and projecting to PVN, DMH, and LH (data not shown).

The AP and NTS of the brainstem are partially spared by postnatal MSG-treatment

We next examined the AP and NTS to determine whether MSG treatment ablates these nuclei to the same extent as the ARC. H&E staining of the AP and NTS shows a less severe change in the gross morphology of the AP and NTS of MSG-treated (Fig. 3 B, D, and F) compared to saline-treated (Fig. 3A, C, and E) mice, indicating that MSG-treatment preferentially ablates the ME and ARC, while largely sparing the AP and NTS. These observations were reconfirmed in MSG-treated female littermates (data not shown).

Peripheral administration of PYY_{3-36} reduces food intake in postnatally MSG-treated mice

We examined the ability of peripherally administered PYY_{3-36} to reduce food intake in MSG-treated mice. We chose a fast-induced refeeding paradigm to assay the effects of PYY_{3-36} on feeding because MSG-treatment causes the loss of the two nocturnal peaks of food intake, normally occurring at the onset of the dark cycle (Stricker-Krongrad et al., 1998). In a 16 h fast-induced refeeding paradigm, 5 μg/100g of PYY_{3-36} injected ip caused a significant reduction of food intake in male and female saline-treated and MSG-treated mice (Fig. 4). IP PYY_{3-36} inhibited food intake for the duration of the experiment

in saline treated male mice (Fig. 4A), as observed previously in wt mice (Halatchev and Cone, 2005). Likewise, peripheral administration of PYY_{3-36} significantly reduced food intake in MSG-treated mice for the duration of the experiment compared to saline administered MSG-treated controls (Fig. 4A and Fig. 4B), although, not to the same degree as in saline-treated mice. At 4 h, for example, PYY_{3-36} injected saline-treated male mice ate 50 ±11% and MSG-treated male mice ate 22 ± 10% compared to their respective saline injected controls (Fig. 4A). Furthermore, the saline injected MSG-treated mice also consumed significantly less food than their saline injected saline-treated littermates (Fig. 4), possibly reflecting a loss of orexigenic drive from NPY neurons ablated by the MSG treatment.

Peripheral administration of PYY_{3-36} causes c-Fos induction in the iNTS of the brainstem but not in ARC of the hypothalamus of MSG-treated mice

Next, we examined c-Fos expression in the residual ARC nucleus of the hypothalamus, and in the NTS of the brainstem in MSG-treated mice (Batterham et al., 2002; Halatchev and Cone, 2005). Nocturnal ip injection (1900 h) of PYY_{3-36} (5 μg/100g) induced c-Fos at similar levels in MSG-treated mice and saline-treated mice, in the NTS (Fig. 5E-L and N) but not in the ARC (Fig. 5C, D, and M). In the NTS of saline-treated mice PYY_{3-36} (5 μg/100g) caused a 4-fold increase in c-Fos expression (Fig. 5E, F, I, J, and N) and a 3-fold increase in c-Fos expression in MSG treated mice (Fig. 5G, H, K, L, and N). MSG-treatment removed any significant induction of c-Fos by PYY_{3-36} in the ARC (Fig. 5C, D, and M) compared to saline-treated mice (Fig. 5A, B, and M).

DISCUSSION

Multiple laboratories have demonstrated that administration of PYY_{3-36} inhibits food intake in multiple species, including mice, rats, monkeys and humans (Adams et al., 2004; Batterham et al., 2002; Challis et al., 2004; Challis et al., 2003; Chelikani et al., 2005; Cox and Randich, 2004; Halatchev et al., 2004; Koda et al., 2005; Moran et al., 2005; Pittner et al., 2004). The initial observation that peripheral PYY_{3-36} induces c-Fos, a marker of neuronal activation, in ~12% of ARC POMC neurons led the authors to further investigate whether the anorexigenic actions of intraperitoneal administration of PYY_{3-36} might be directly mediated via the ARC NPY/AgRP and POMC circuitry (Batterham et al., 2002). In this same study, bath applied PYY_{3-36} was reported to increase the firing of POMC neurons, presumably indirectly by inhibiting presynaptic release of GABA, an inhibitory neurotransmitter, from tonically active NPY neurons (Batterham et al., 2002). This presynaptic effect, inhibiting GABA release from NPY terminals, was postulated to be mediated via Y2 presynaptic autoinhibitory receptors, for which PYY_{3-36} shows high affinity. Finally, in a hypothalamic explant, PYY_{3-36} reduced the release of NPY and increased the released of α-MSH. Thus a mechanism for the anorexigenic actions of PYY_{3-36} was proposed, in which peripherally released PYY_{3-36} accesses the ARC and reduced GABAergic tone onto POMC cells by binding to Y2 receptors on NPY terminals. The inhibition of NPY neurons and concomitant activation of POMC neurons in the ARC reduces the orexigenic drive and produces a net anorexigenic drive leading to a reduction in food intake.

However, there has been a considerable amount of controversy surrounding this peptide, and the proposed mechanism of action, including difficulty, by some labs to reproduce the reduction in food intake (Tschop et al., 2004). MC4-$R^{-/-}$ (Halatchev et al., 2004), Agouti (Martin et al., 2004), and $POMC^{-/-}$ (Challis et al., 2004) mice all show equal reduction of food intake in response to peripheral PYY_{3-36} administration, arguing that neither the POMC gene nor the melanocortin receptors are required for the anorexigenic actions of PYY_{3-36}.

One remaining possibility, from the original hypothesis, is that instead of producing a net anorexigenic drive from the ARC nucleus, PYY_{3-36} may instead decrease an existing orexigenic drive to cause a reduction of food intake. In this report we sought to determine whether instead of utilizing the POMC arm of the central melanocortin circuitry, peripheral PYY_{3-36} may exert its effects by reducing orexigenic drive via NPY/AgRP neurons. To address this question our collaborator, Dr. Su Qian, examined the ability of ip PYY_{3-36} to reduce food intake in NPY/AgRP double knockout mice, generated by crossing $NPY^{-/-}$ mice with $AgRP^{-/-}$ mice (Qian et al., 2002). PYY_{3-36} dose dependently and significantly reduced food intake in NPY/AgRP mice (Fig. 1). This result indicates that neither NPY nor AgRP are essential for the anorexigenic actions of peripheral PYY_{3-36}. Therefore, anorexigenic effects of PYY_{3-36} do not occur via reduction of the pre-existing NPY or AgRP orexigenic drive. In fact, PYY_{3-36} shows a greater effect on food intake, relative to saline treatment, in NPY/AgRP double knockout mice than in their age and sex matched controls (Fig. 1). This result may indicate that

tonic NPY/AgRP orexigenic tone may counteract the anorexigenic effects of PYY_{3-36}. Intriguingly, $NPY^{-/-}$ mice also show hypersensitivity to long-acting anorexigenic factors, like leptin, showing greater suppression of food intake and body weight following peripheral administration (Erickson et al., 1996a; Hollopeter et al., 1998a), but have unaltered response to other short-acting feeding inhibitors, such as corticotrophin releasing factor (CRF), and MTII, a melanocortin –4 receptor agonist (Hollopeter et al., 1998b). Finally, the observation that NPY/AgRP peptides are not essential for the anorexigenic actions of peripheral PYY_{3-36} may indicate that activating the Y2 receptor may have effects on systems other than NPY. In a recent report we showed that peripheral PYY_{3-36} produces conditioned taste aversion, presumably by acting in the AP and NTS of the brainstem (Halatchev and Cone, 2005), indicating that the Y2 receptor may be involved in mediating some aversive effects. Future studies are needed to determine if peripheral PYY_{3-36} can produce conditioned taste aversion in Y2 receptor knockouts since they seem to be insensitive to the satiating effects of high fat food (Naveilhan et al., 1999).

The dorsal vagal complex (DVC) of the brainstem is a major brain site involved in the integration and regulation of meal size, receiving vagal afferents and humoral signals. The NTS, AP, and dorsal motor nucleus of the vagus (DMN) together constitute the DVC. Numerous gut peptides, like CCK and ghrelin, have been shown to mediate or relay their effects on food intake via the DVC. The brainstem has been shown to be sufficient to mediate responses involving taste and meal size in the decerebrate animal (Grill and Kaplan, 1992; Grill and Smith, 1988; Seeley et al., 1994). It was, therefore,

seemingly uncharacteristic for a gut released peptide, like PYY_{3-36}, to act directly at the ARC of the hypothalamus to relay satiety information and reduce food intake. Indeed, peripheral administration of PYY_{3-36}, dose dependently activates c-Fos in neurons of the AP and NTS (Halatchev and Cone, 2005) and vagotomy in the rat abolishes the c-Fos induction in the ARC (Koda et al., 2005), suggesting that the brainstem may be the primary site of action of the peptide. In this report we investigated whether the ARC of the hypothalamus is required for the anorexigenic actions of peripherally administered PYY_{3-36}. We chose the postnatal MSG treated mouse model, which results in the chemical ablation of the ARC nucleus, to evaluate the effectiveness of peripheral PYY_{3-36} to reduce food intake and induce c-Fos immunoreactivity in the absence of an intact ARC. Postnatal MSG treatment caused marked ablation of the ME and the ARC nucleus of the hypothalamus with relative sparing of the AP and the NTS of the brainstem (Fig. 2 and 3). The disruption of numerous neuroendocrine systems in the ARC of the hypothalamus caused a distinctive phenotype, exemplified by stunted physical growth, hypersensitivity to stress, disruption in meal patterns, and increase in body fat (Meister et al., 1989; Stricker-Krongrad et al., 1998). Furthermore, immunohistochemical examination of the ARC nucleus of MSG-treated mice revealed a complete loss of any detectable NPY neurons and greater than 95% reduction in POMC neurons (Fig. 2). This substantial loss of ARC NPY and POMC neurons in some respects represents a selective ARC NPY and POMC neuronal knockout, with some notable differences, like the loss of other neuronal factors such as GABA, glutamate, etc. co-expressed in NPY and POMC neurons.

In a 16 h fast-induced refeeding paradigm, ip PYY_{3-36} treatment significantly reduced the food intake of both male and female MSG-treated mice, compared to saline injected MSG-treated controls (Fig. 4). However, peripheral administration of PYY_{3-36} was less effective at reducing food intake in MSG-treated mice compared to saline-treated wt mice (Fig. 4). These results indicated that even though the ARC is not essential for the anorexigenic actions of peripheral PYY_{3-36}, it may play a role downstream of the brainstem. Alternatively, the reduced efficacy of PYY_{3-36} to reduce food intake in MSG-treated mice may be due to the substantial neuroanatomical and neuroendocrine abnormalities associated with MSG ablations (Bergen et al., 1999; Meister et al., 1989; Reyes and Sawchenko, 2002; Stricker-Krongrad et al., 1998). MSG-treated mice were hypersensitive to stress, requiring much longer acclimatization to the feeding protocol with repeated fasting (data not shown, and (Dawson and Lorden, 1981)), and ate significantly less (<50%) after 16 h fast than saline-treated wt controls (Fig. 4). Indeed, we previously showed that the anorexigenic effects of PYY_{3-36} could be masked by stress due to a significant reduction in basal food intake (Halatchev et al., 2004). Additionally, MSG-treated mice may have a reduced orexigenic drive to eat after a 16 h fast, an effect also seen in $NPY^{-/-}$ mice (Bannon et al., 2000) due to absence of ARC NPY neurons. Finally, MSG-treated mice may be viewed as a post-developmental ARC neuronal knockout and as such confirming previous observations in NPY/AgRP double knockout (Fig. 1), $MC4\text{-}R^{-/-}$ (Halatchev et al., 2004), Agouti (Martin et al., 2004), and $POMC^{-/-}$ (Challis et al., 2004) mice showing reduction of food intake with peripheral PYY_{3-36}.

Previous reports have shown that peripheral administration of PYY_{3-36} induces c-Fos in both the ARC and the NTS, two areas known to be involved in the integration and regulation of energy homeostasis (Halatchev and Cone, 2005). Furthermore, vagotomy abolishes the ARC c-Fos induced by peripheral PYY_{3-36} (Koda et al., 2005), indicating that the ARC c-Fos may be induced transynaptically via the brainstem. In this study we used c-Fos induction as a functional neuroanatomical marker and examined the neuronal activation in the ARC and NTS following peripheral administration of PYY_{3-36} in control and MSG-treated mice. In control treated mice a non-significant increase in ARC c-Fos was observed (Fig. 5), however this would likely become significant with an increased n-number. However, other reports have previously shown a significant induction of c-Fos over the basal state with peripheral injections of PYY_{3-36} (Batterham et al., 2002; Koda et al., 2005). Postnatal MSG treatment drastically reduced the c-Fos levels in the ARC in the basal, saline treated, state and completely ablated the trend for c-Fos induction with peripheral PYY_{3-36} treatment, observed in the postnatal saline-treated mice (Fig. 5). The lack of c-Fos induction in the ARC of MSG-treated mice may be a reflection of the marked hypocellularity produced by postnatal MSG treatment (Fig. 2). Indeed, in the NTS, where relatively less severe ablation was observed after postnatal MSG-treatment (Fig. 3), the same basal levels of c-Fos were observed after saline treatments in postnatally saline and MSG-treated mice (Fig. 5). As reported previously (Halatchev and Cone, 2005), peripheral treatment with 5 μg/100g of PYY_{3-36} caused a 4-fold increase in c-Fos in the intermediate NTS (iNTS) of the brainstem (Fig. 5). Likewise, ip PYY_{3-36} caused a significant c-Fos induction in the iNTS of MSG-treated mice, albeit, at lower levels than saline-treated mice, a 3-fold increase over baseline in MSG-treated mice

compared to a 4-fold over baseline in saline-treated mice (Fig. 5). The fact that peripheral PYY_{3-36} retained its ability to reduce food intake and activate c-Fos in neurons of the iNTS but not in the chemically ablated ARC indicates that the ARC nucleus may not be essential for the anorexigenic effects of PYY_{3-36}. A limitation of this study is the fact that c-Fos is only a marker of neuronal activation and not inhibition, thus there may be yet unknown neuronal populations inhibited by PYY_{3-36} that contribute to its mechanism of action.

Furthermore, these observations point to the possibility that the brainstem and in particular the iNTS may be one of the first sites of action for peripherally administered PYY_{3-36}. With the development of a chronic decerebrate rat model (Flynn and Grill, 1988; Grill and Kaplan, 1992; Grill and Norgren, 1978a; Grill and Smith, 1988; Seeley et al., 1994), lacking a forebrain-brainstem link, the brainstem has been shown to be sufficient to control meal size. Therefore, it is possible that sites in the brainstem may be sufficient to mediate the anorexigenic effects of peripherally secreted PYY_{3-36}.

Finally, we attempted to examine the neurochemical identity of the c-Fos neurons induced by peripheral PYY_{3-36} (Appendix Fig. 5). We hypothesized that PYY_{3-36} may mediate part of its anorexigenic affect through the catecholaminergic (tyrosine hydroxylase [TH] neurons in the NTS as the neuroanatomical position of c-Fos activation appeared to be similar to the A2 catecholaminergic population and NTS TH neurons are activated by other gut peptides, such as CCK (Monnikes et al., 1997). However, nocturnal peripheral administration of PYY_{3-36} did not increase the number of TH

neurons expressing c-Fos compared to saline treatment (Appendix Fig. 5). This observation indicates that the anorexigenic and aversive effects of peripheral PYY_{3-36} might not be mediated through the NTS TH neurons.

In summary, in this report we sought to determine whether or not NPY/AgRP neurons and an intact ARC nucleus are essential to mediate the anorexigenic effects of peripheral PYY_{3-36}. We tested the ability of ip injected PYY_{3-36} to reduce food intake in NPY/AgRP double knockout mice and in postnatally MSG-treated mice. PYY_{3-36} reduced food intake in both mouse models indicating that neither the NPY/AgRP peptides, nor even the arcuate nucleus are essential to mediate its actions. Furthermore, postnatal MSG-treatment abolished the induction of c-Fos, a marker of neuronal activation, in the ARC but not in the iNTS, indicating that activation of iNTS neurons occurs independently of ARC neurons and may be one of the first sites for the integration and possibly the mediation of the anorexigenic effects of PYY_{3-36}. Finally, peripheral PYY_{3-36} does not activate c-Fos in NTS TH neurons, indicating its anorexigenic actions are not mediated by the A2 catecholaminergic neurons.

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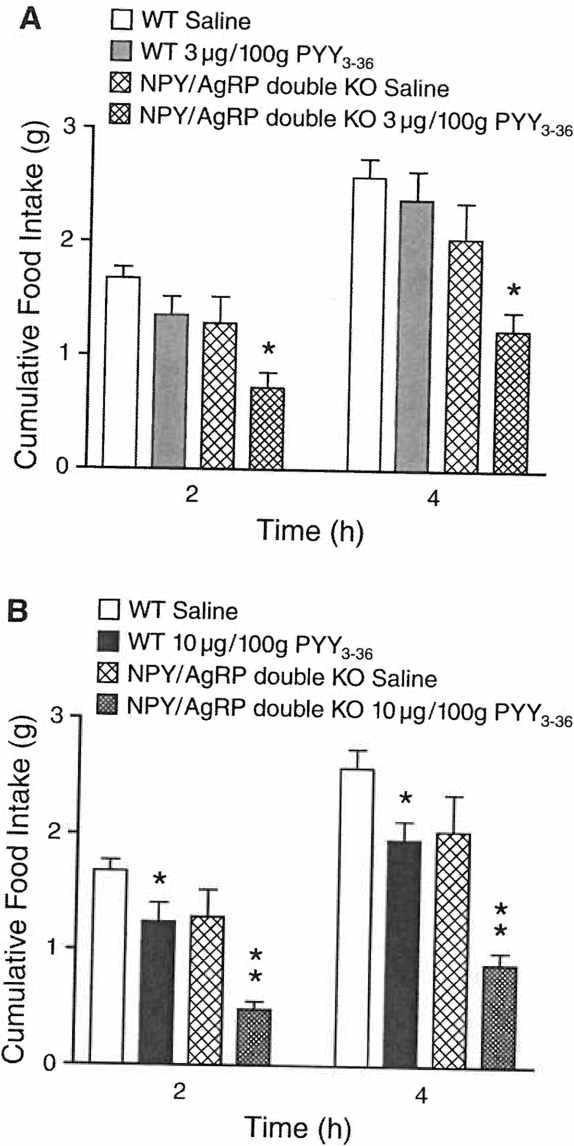

Fig. 1. Peripheral PYY_{3-36} dose dependently inhibits food intake in NPY/AgRP double knockout and WT mice. A, 16 h fast induced refeeding response of WT and NPY/AgRP double knockout mice to a PYY_{3-36} dose of 3 µg / 100 g at 2 and 4 h post injection (n=12, each group). B, 16 h fast induced refeeding response of WT and NPY/AgRP double knockout mice to a PYY_{3-36} dose of 10 µg / 100 g at 2 and 4 h post injection (n=12, each group). Data are expressed as mean ± SEM, statistics by two-way ANOVA followed by Student's (two-tailed) t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

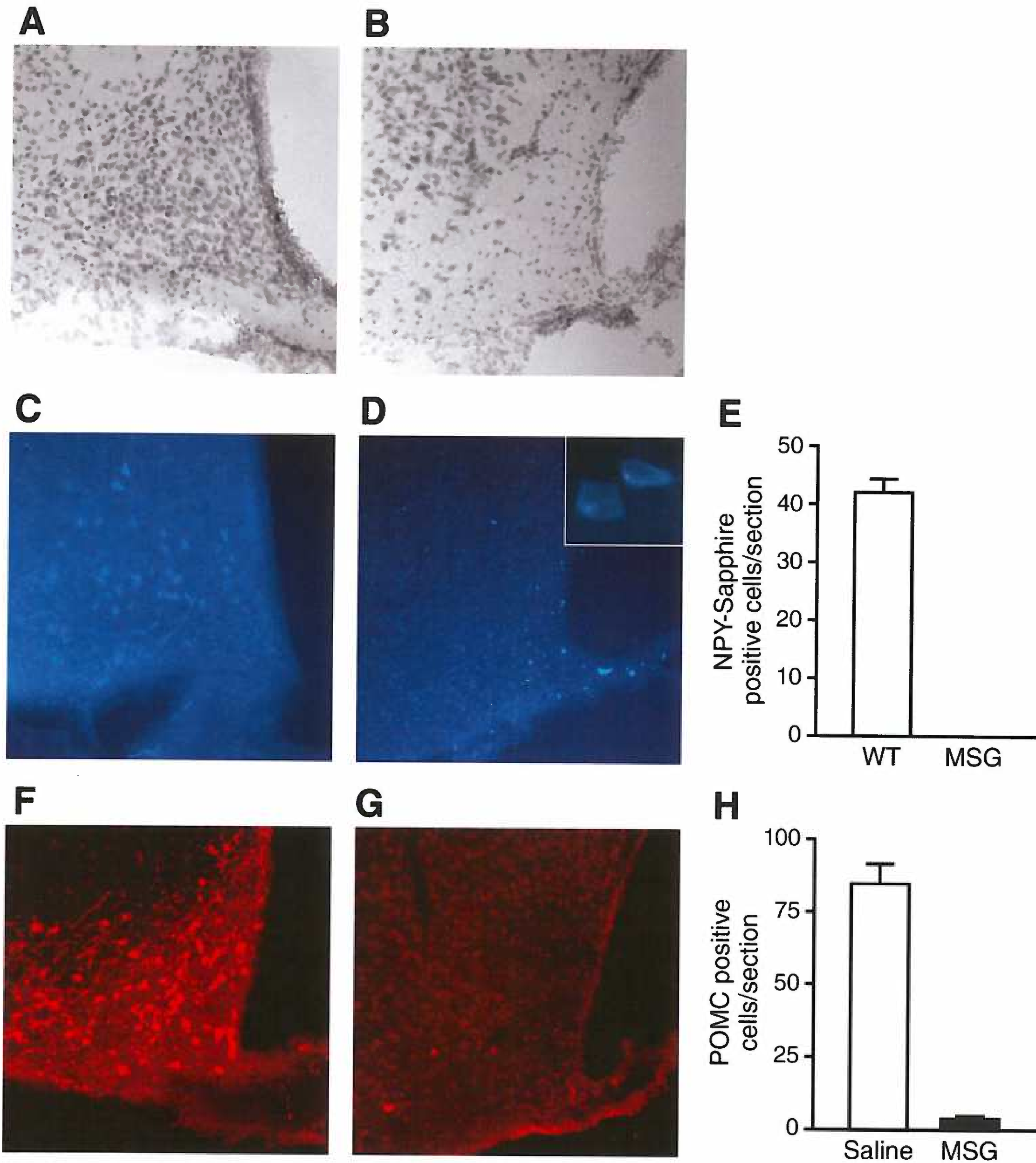

Fig. 2. Postnatal MSG treatments cause a marked reduction of neurons within the arcuate nucleus, including NPY and POMC neurons. A, H&E staining of the ARC of a saline-treated mouse. B, H&E staining of the ARC of a MSG-treated mouse. C, NPY-sapphire autofluorescence in the ARC of WT NPY-sapphire mouse. D, NPY-sapphire autofluorescence in the ARC of MSG-treated NPY-sapphire mouse. E, Quantization of NPY-sapphire autofluorescence in WT and MSG-tread NPY-sapphire mice. F, POMC immunoreactivity in the ARC of saline-treated mouse. G, POMC IR in the ARC of MSG-treated mouse. H, Quantization of POMC immunoreactivity in saline-treated and MSG-treated mice. Data are expressed as mean ± SEM.

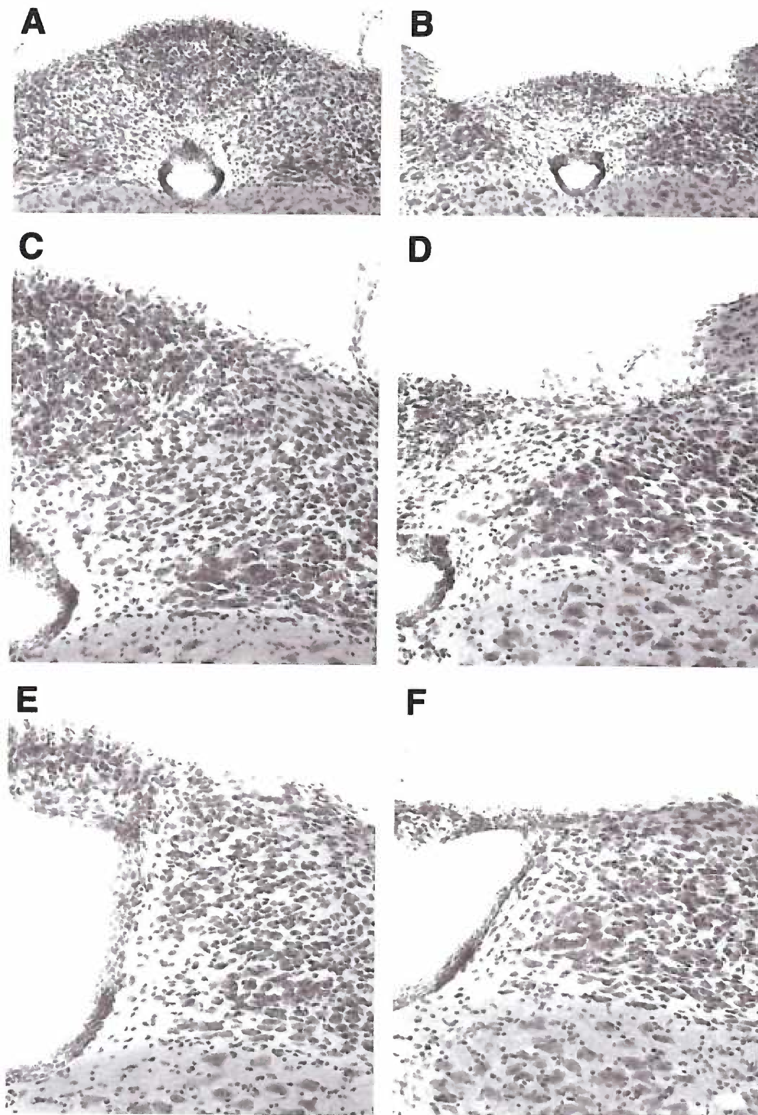

Fig. 3. Postnatal MSG treatments cause a less severe reduction of AP and NTS neurons. A and C, H&E stain for NTS at the level of the AP in saline-treated mouse, where (B) and (D), are for MSG-treated mouse. E, H&E staining of the NTS at the level of the 4th ventricle in saline-treated mouse. F, H&E staining of the NTS at the level of the 4th ventricle in MSG-treated mouse.

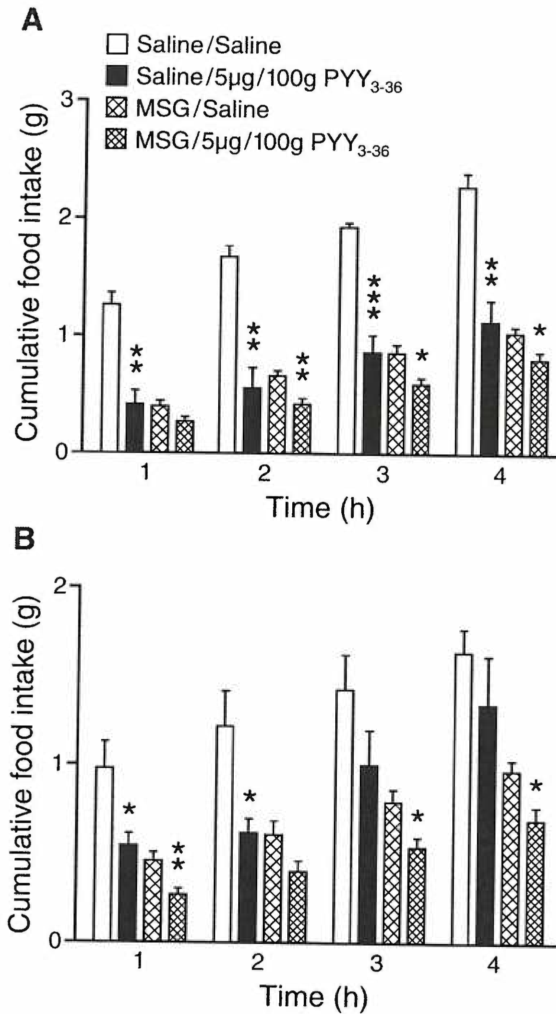

Fig. 4. Peripheral PYY_{3-36} inhibits food intake in male and female MSG-treated mice. A, 16 h fast induced refeeding response of male saline-treated and MSG-treated mice to a PYY_{3-36} dose of 5 µg / 100 g at 1, 2, 3, and 4 h post injection (saline-treated mice, n=4; MSG-treated mice, n=7). B, 16 h fast induced refeeding response of female saline-treated and MSG-treated mice to a PYY_{3-36} dose of 5 µg / 100 g at 1, 2, 3, and 4 h post injection (saline-treated mice, n=4; MSG-treated mice, n=7). Data are expressed as mean ± SEM, statistics by two-way ANOVA followed by Student's (two-tailed) t-test: *, $P<0.05$; **, $P<0.01$; ***,$P<0.001$.

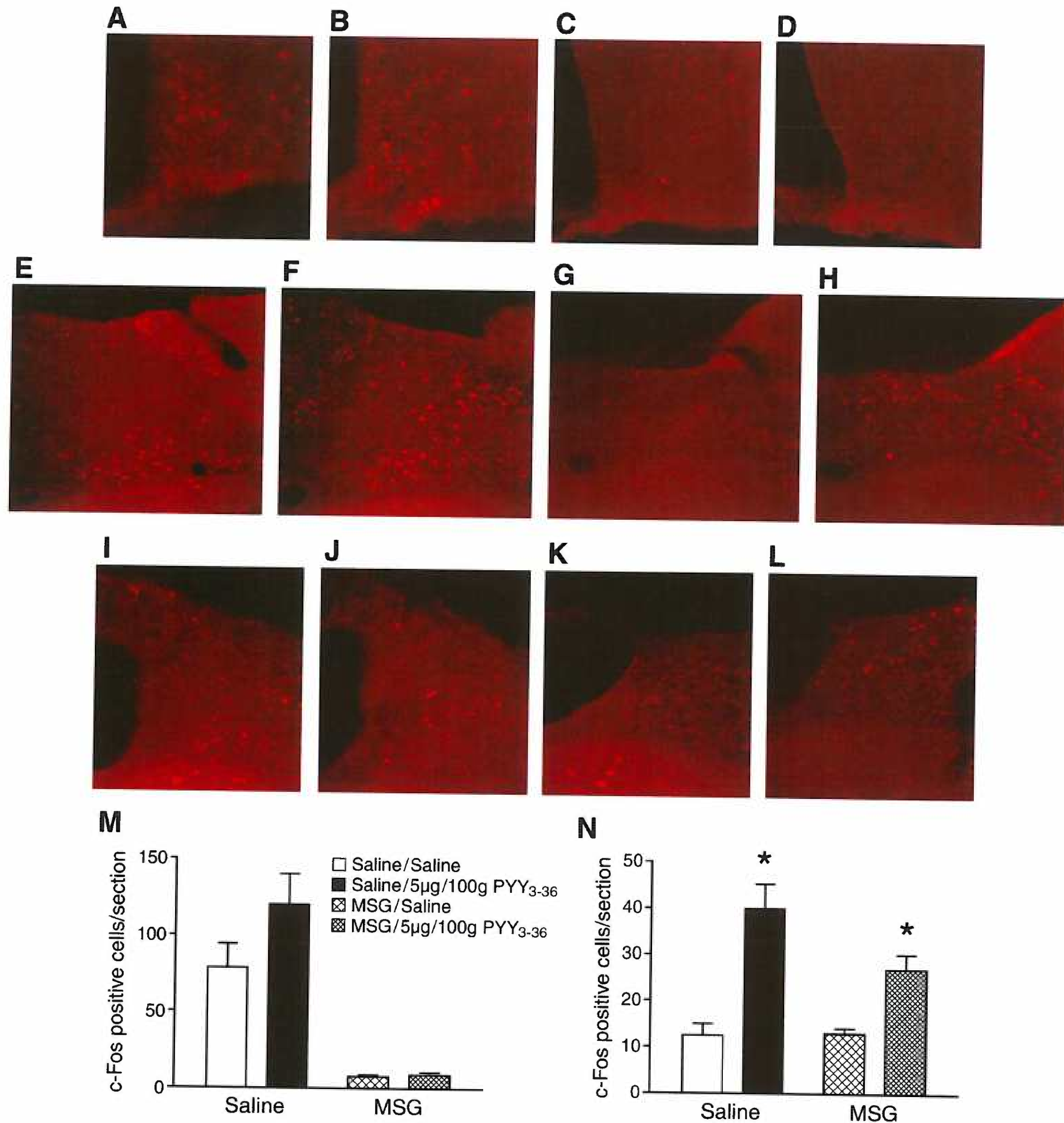

Fig. 5. Postnatal MSG-treatment diminishes c-Fos expression in the ARC but not in the iNTS. c-Fos expression in the ARC of saline-treated mouse injected with either ip saline (A), or 5 µg / 100 g dose of PYY_{3-36} (B). c-Fos expression in the ARC of MSG-treated mouse injected with either ip saline (C), or 5 µg / 100 g dose of PYY_{3-36} (D). In the NTS, at the level of AP, ip PYY_{3-36} (5 µg / 100 g) induces c-Fos expression in saline-treated (F) and MSG-treated mice (H) compared to saline treated controls of either postnatal treatment group (E) and (G), respectively. In the NTS, at the level of the 4th ventricle, ip PYY_{3-36} (5 µg / 100 g) induces c-Fos expression in saline-treated (J) and MSG-treated mice (L) compared to saline treated controls of either postnatal treatment group (I) and (K), respectively. M, Quantization of c-Fos IR in the ARC (M) and the iNTS (N) of saline-treated and MSG-treated mice after ip administration of saline (saline-treated, n=2; MSG-treated, n=3) or PYY_{3-36} at the dose of 5 µg / 100 g (saline-treated, n=2; MSG-treated, n=4). Data are expressed as mean ± SEM, statistics by Student's (two-tailed) t-test: *, $P<0.05$.

SUMMARY AND CONCLUSIONS

Peptide YY_{3-36} (PYY_{3-36}), a peptide released postprandially by the gut, has been demonstrated to inhibit food intake in rodents (Batterham et al., 2002). This peptide has achieved additional attention due to the observation that continuous intravenous infusion of PYY_{3-36} reduced 24 h food intake and hunger scores in a small number of lean and obese human subjects, suggesting a potential clinical utility of the peptide in the treatment of obesity (Batterham et al., 2003; Batterham et al., 2002). Little is known about the mechanism by which PYY_{3-36} inhibits food intake, although the peptide was initially thought to inhibit food intake by producing a net anorexigenic drive from the ARC melanocortin system (Batterham et al., 2002). However, in nocturnal and fast-induced refeeding paradigms we found that MC4-R knockout (Chapter 1), and MC3-R knockout mice (Appendix Fig. 2) were equally responsive to the anorexigenic effects of PYY_{3-36} as wild type sex and age matched controls.

The main focus of my thesis was to elucidate the mechanism of the anorexigenic actions of PYY_{3-36}. However, understanding the physiology of PYY_{3-36} was further complicated by the fact that 12 collaborating laboratories had difficulty demonstrating inhibition of feeding by the peptide in rodents (Tschop et al., 2004). In this thesis we demonstrate that, like CCK, PYY_{3-36} dose-dependently inhibits food intake by approximately 20–45% over a 3 to 4 h period post ip administration, with no effect on 12 h food intake. This short-lived satiety effect is not seen in animals that are not thoroughly acclimated to handling and ip injection, thus potentially explaining the difficulty in reproducing the effect (Chapter 1). Since then, seven independent laboratories have confirmed the ability of

PYY_{3-36}, administered by various routes, to cause a short-term reduction of food intake in multiple animal species, including mice, rats, and monkeys (Adams et al., 2004; Challis et al., 2004; Challis et al., 2003; Chelikani et al., 2005; Cox and Randich, 2004; Halatchev et al., 2004; Koda et al., 2005; Moran et al., 2005; Pittner et al., 2004). Furthermore, the senior author of the Tschop et al., has mow recanted his statement that PYY_{3-36} does not produce a short-term inhibition of food intake (Gura, 2004).

Likewise the mechanism of anorexigenic action of PYY_{3-36} has been brought into question. In agreement with our observation that the anorexigenic effects of PYY_{3-36} are not dependent on melanocortin signaling (Chapter 1 and Appendix Fig. 2), Agouti (A^y) (Martin et al., 2004), and $POMC^{-/-}$ (Challis et al., 2004) mice have also been shown to have a comparable reduction in food intake in response to peripheral PYY_{3-36} administration to wild-type mice, arguing that neither the POMC gene nor the melanocortin receptors are required for the anorexigenic actions of PYY_{3-36}. One remaining possibility, derived from the original hypothesis, was that instead of producing a net anorexigenic drive through melanocortin signaling, PYY_{3-36} may instead decrease existing NPY/AgRP orexigenic drive from the ARC to produce a reduction of food intake. However, PYY_{3-36} dose dependently and significantly reduced food intake in NPY/AgRP double knockout mice (Chapter 3) in a 16 h fast induced refeeding paradigm. These observations indicate that neither NPY nor melanocortin signaling are essential for the anorexigenic actions of peripheral PYY_{3-36}. Indeed, we also demonstrated that ARC ablation by post-natal MSG treatment does not block the inhibition of feeding by PYY_{3-36} (Chapter 3), arguing that the ARC is not essential for the anorexigenic effects of PYY_{3-36}.

In this thesis we sought to identify an alternative mechanism of action for PYY_{3-36} in the regulation of food intake. We have thus proposed that peripheral PYY_{3-36} may have direct effects in the AP and iNTS of the brainstem to cause aversion in mice. This hypothesis is supported by several observations. Firstly, vagotomized mice are responsive to the anorexigenic effects of peripherally administrated PYY_{3-36} (Chapter 2). Secondly, dose-dependent activation of AP and iNTS neurons was observed with increasing concentrations of PYY_{3-36}, as assayed by the expression of c-Fos, a marker of neuronal activation, where the minimum dose shown to inhibit food intake caused a significant induction of c-Fos in the iNTS (Chapter 2). Thirdly, peripheral PYY_{3-36} caused conditioned taste aversion to a flavor paired with PYY_{3-36} treatment, at doses that reliably and reproducibly inhibit short-term food intake (Chapter 2). Finally, in mice treated postnatally with MSG, which have an ablated ARC nucleus, peripheral PYY_{3-36} reduced food intake and induced c-Fos activation in the iNTS but not in the ARC (Chapter 3). This observation suggests that the iNTS, in the absence of the ARC, is adequate to mediate the anorexigenic effects of PYY_{3-36}. Further studies are needed to establish whether the NTS and AP are required and sufficient to mediate the anorexigenic actions of peripheral PYY_{3-36}.

This thesis work may provide some insights into whether PYY_{3-36} is a physiological gut released peptide regulating satiety. In his original report, identifying CCK as the archetypal gut-released satiety peptide, Smith proposed a set of criteria to classify a gut peptide as a satiety factor ((Smith et al., 1981), and Introduction). In short, the following

criteria were proposed: 1) the factor/hormone has to be released/activated in response to feeding, 2) exogenous administration of the factor/hormone should cause a dose dependent inhibition of feeding, 3) the factor/hormone's anorexigenic effect should be of rapid onset and short-lived to respond to ingestive behavior, 4) the factor/hormone should be effective at physiological (post-meal released concentrations) concentrations to inhibit feeding, and 5) the anorexigenic effect of the factor/hormone should not be as a result of illness, like aversion. Indeed while PYY_{3-36} fits many of the criteria of satiety factor, see below, there are some notable differences.

1. Ingestion of food, especially fatty acids, and proteins, induces the release of PYY_{3-36} from L-cells of the ileum and colon (Bottcher et al., 1993; McDonald et al., 1993). The increase of plasma PYY is observed shortly after ingestion of food, presumably due to vagally mediated release (Zhang et al., 1993b) but, unlike CCK, peak levels are not reached for 60-90 min (Taylor, 1985).

2. In addition to work presented in this thesis, numerous reports by independent groups have shown that peripheral administration of PYY_{3-36} can produce a dose dependent inhibition of food intake (Adams et al., 2004; Challis et al., 2004; Challis et al., 2003; Chelikani et al., 2005; Cox and Randich, 2004; Koda et al., 2005; Pittner et al., 2004).

3. Like CCK, the onset of the anorexigenic actions of PYY_{3-36} is very rapid, reducing food intake up to 50% in the first half an hour of measurement (Adams et al.,

2004). However, the duration of the anorexigenic actions of PYY_{3-36} has been shown to vary depending on the report, the route of administration and the species studied. For example, PYY_{3-36} significantly reduces food intake for as little as 2 h post ip injection in mice (Halatchev et al., 2004) and as long as 24 h after 90 min iv infusion in humans (Batterham et al., 2003; Batterham et al., 2002). A possible explanation for this astounding difference may be that an effective iv concentration is required for the anorexigenic actions of PYY_{3-36}. In their report Chelikani et al. showed that while 15 min iv infusion of PYY_{3-36} produces short-lasing reduction of food intake, 3 h constant iv infusion, of the same total dose, causes a long-lasting inhibition of food intake (Chelikani et al., 2005). Indeed, ip administration of PYY_{3-36}, which may result in lower plasma levels due to first pass effect of the liver, reduces food intake only for a short time ((Adams et al., 2004; Challis et al., 2004; Challis et al., 2003; Cox and Randich, 2004; Koda et al., 2005; Pittner et al., 2004) and Chapter 1, 2, and 3). Additional experiments are necessary to examine if prolonged elevation of plasma PYY_{3-36} concentration may results in loss of its the anorexigenic effects due to receptor internalization or desensitization.

4. The current understanding of whether PYY_{3-36} can reduce food intake at the physiological postprandial concentration is limited due to the lack of a reliable assay to measure plasma PYY_{3-36} levels. The techniques used currently are incapable of differentiating between the two endogenous forms of the peptide, the less potent anorexigenic PYY_{1-36} and more anorexigenic PYY_{3-36}. In the original paper by Batterham et al. the peak postprandial total PYY levels in the rat were measured to be

112.1± 7.8 pmol L^{-1} equating to ~0.3µg/100g ip (Batterham et al., 2002), determined by measuring peak plasma PYY_{3-36} concentrations following ip administration. Indeed, they further showed that PYY_{3-36} administered at the physiological postprandial level either ip in rats or iv in humans, significantly reduced food intake (Batterham et al., 2002). However, the ability of PYY_{3-36} to reduce food at that dose is dependent on the route of administration and species studied. For example, IV infusion of postprandial concentrations of PYY_{3-36} reduces food intake in humans and rats while the same concentration administered ip in mice does not reduce food intake significantly (Halatchev et al., 2004; Pittner et al., 2004). This may either be due to the first pass effects of the liver, the effective plasma concentration reached following ip administration or interspecies variability. Indeed, mice have much higher metabolic rate than rats or humans, therefore, lower concentrations of PYY_{3-36} may be reaching the circulation after ip injection in mice.

5. In the literature there is often a large distinction made between satiety and aversion. However, while ingested toxins, like LiCl, may reduce or halt food intake through purely aversive mechanisms, mediated by brainstem chemoreceptors, other endogenous substances, like CCK and glucagons-like peptide-1 (GLP-1), reduce food intake through homeostatic mechanisms where "aversion" may be equated with extreme satiety (Verbalis et al., 1986). We have all eaten one too many slices of pizza and felt uncomfortable due to extreme fullness but that feeling is very different than having eaten a bad oyster, resulting in food poisoning, vomiting and the avoidance of that food in future. Indeed, there is a body of literature showing that aversion induced by high doses

of satiety factors, like CCK and GLP-1, as well as non-homeostatic forms of aversion, like LiCl, may act via the same circuits in the AP and NTS.

We have shown that ip administration of PYY_{3-36} dose dependently induces c-Fos expression in the AP and intermediate NTS (Chapter 2) in a pattern very similar to LiCl (Schafe et al., 1995). Furthermore, doses of ip PYY_{3-36} that reliably and reproducibly inhibit food intake cause conditioned taste aversion in mice (Chapter 2) thus suggesting that PYY_{3-36} may not be acting as a satiety factor. Additional evidence that PYY may cause aversion comes from pathophysiological conditions presenting with elevated plasma PYY levels. Indeed, serum elevation of systemic PYY_{1-36} due to a long-standing type I diabetes in humans is associated with gastrointestinal symptoms, such as nausea, vomiting, and diarrhea (El-Salhy and Sitohy, 2001). Like wise, gastric bypass surgery or certain kinds of gastrointestinal tract inflammatory disease and cancers, present with reduction in food intake and subsequent anorexia which may due to elevation of plasma PYY_{1-36} levels associated with these conditions (Batterham et al., 2002; Naslund et al., 1997). However, even the prototypical gut released satiety factor, CCK, has been shown to cause conditioned taste aversion at high doses (Deutsch and Hardy, 1977; Ervin et al., 1995) and activates neurons in the AP and intermediate NTS. This may be an indication that satiety and aversion are on a continuum, with short-term meal termination at one end and long-term avoidance on the other.

However, perhaps the most intriguing and revealing evidence that PYY may cause emesis comes from studies in dogs. Importantly, a very strong correlation between

emesis and plasma PYY concentrations was observed in dogs (Harding and McDonald, 1989), in which PYY caused vomiting at concentrations lower than 120 pmol/kg with an ED_{50} of 325 pmol/kg. The emesis could further be reversed by AP ablations (Harding and McDonald, 1989) or by administration of either ondansetron (Perry et al., 1994) or granisetron, highly selective serotonin 3 receptor (5-HT3-R) antagonists. Future studies are necessary to determine if like the emetic effects of PYY, the anorexigenic actions of PYY_{3-36} are mediated by the central serotonin system, and in particular the 5-HT3-R. For example, determining if peripherally administered PYY_{3-36} can reduce food intake in 5-HT3-R knockout mice or after central administration of 5-HT3-R specific antagonist.

Although considerable effort has been undertaken in the past few decades to develop a drug capable of reducing food intake or body weight, there is still a need for better therapeutic agents. Drugs like phentermine and fenfluramine (Phen-Fen), which produced a significant reduction in body weight were pulled from the market due to toxic side effects, such as primary pulmonary hypertension. Therefore, strong interest exists in developing a drug, which is highly tolerated, producing minimal side effects, and capable of reducing body weight. Previous clinical experience shows that replacement or administration of an endogenous compound is usually well tolerated with minimal side effects. Indeed, one possible approach is to identify an endogenous hormone modifying the appetite to cause changes in the subjective feelings of hunger and fullness. Drugs capable of inducing satiety are considered 'appetite suppressants' and may have effects on hypothalamic neuropeptide or neurotransmitter levels. PYY_{3-36} represents one such target candidate. Indeed, continuous iv infusion of PYY_{3-36} in a small number of lean

and obese human subjects reduced their 24 h food intake and hunger scores, suggesting a potential clinical utility of the peptide in the treatment of obesity (Batterham et al., 2003; Batterham et al., 2002). Even though these studies provide an indication of the therapeutic potential of PYY_{3-36} in the treatment of obesity there are also some notable limitations. Firstly, the original studies in humans suggesting a clinical utility of the peptide have not yet been reproduced by another laboratory, and only assayed the ability of PYY_{3-36} to reduce food intake in a small cohort of human subjects. There are various examples in the literature showing that large scale clinical studies are necessary to assess the potency of a drug in a heterogeneous population of subjects. Second, the studies, due to a single dose with a single application paradigm, were not structured to carefully examine side effects, which may result from consecutive administrations or different doses. Indeed, previous attempts with other appetite suppressing agents, like CCK, have shown considerable side effects, which may impede compliance, like abdominal cramping and nausea (Stacher, 1985). Third, although, both of the human studies examined the ability of PYY_{3-36} to reduce of intake in humans neither looked at the ability of the peptide, with chronic or single, administration to reduce body weight. Therefore, future large-scale studies, paying close attention to any undesired side effects, like nausea, and assaying the efficacy to reduce body weight are necessary to assess the clinical utility of PYY_{3-36} in the treatment of obesity.

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APPENDIX

Human and rat PYY_{3-36} reduce food intake equally

The initial report by Batterham and coworkers showed that PYY_{3-36} can inhibit food intake in multiple species, including mice, rats and humans (Batterham et al., 2002). However, there has been a considerable amount of controversy about the ability of the peptide to reduce food intake. In a single, 12 lab collaborative, report the anorexigenic actions of peripherally administered PYY_{3-36} were unable to be reproduced (Tschop et al., 2004). We postulated that the difficulties of some labs to reduce food intake with ip PYY_{3-36} may be due to the species of the peptide injected, rat PYY_{3-36} (R-PYY_{3-36}) vs. human PYY_{3-36} (H-PYY_{3-36}), in to rodents. Indeed, the sequences R-PYY_{3-36} and H-PYY_{3-36} are different by two amino acids, suggesting that they may have different receptor specificity and effects on food intake, like PYY_{1-36} and PYY_{3-36} (for review see (Keire et al., 2002). We assessed the ability R-PYY_{3-36} and H-PYY_{3-36} (3 μg/100g) to reduce food intake in acclimated mice in a 16 h fast induced refeeding paradigm (described in Chapter 1 Methods). Both R-PYY_{3-36} and H-PYY_{3-36} (3 μg/100g) reduced food intake equally at each time point of the experiment (Fig. 1). At 1 h R-PYY_{3-36} reduced food intake by 42 ±12% and H-PYY_{3-36} by 38 ±10% (Fig. 1). Human and rat PYY_{3-36} reduce fast-induced re-feeding to the same extent and with the same time course in acclimated mice, indicating that the differences observe in the ability of PYY_{3-36} to reduce food intake are not dependent on the species of peptide used.

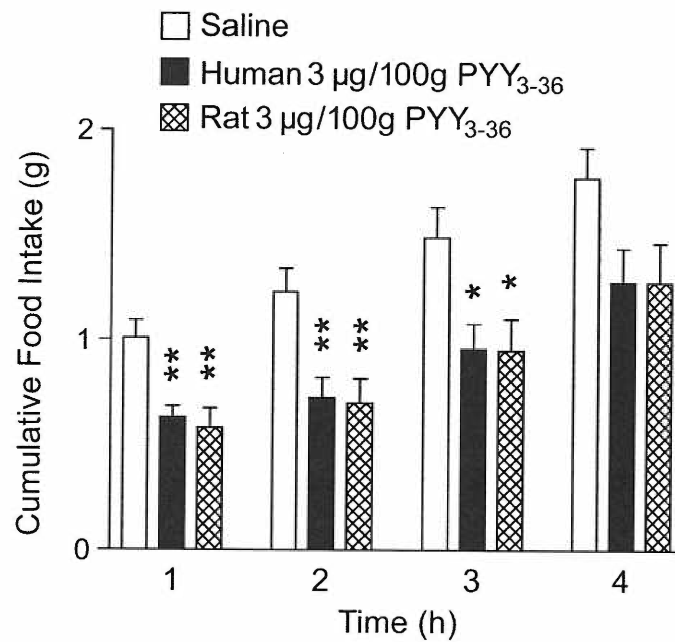


Fig. 1. Human and rat PYY_{3-36} reduce fast-induced re-feeding to the same extent and with the same time course in acclimated mice in a 16 h fast induced refeeding protocol (n=10 for each group) (Methods described in Chapter 1). Data are expressed as mean ± SEM, statistics by two-way ANOVA followed by Student's (two-tailed) t-test: *, $P<0.05$; **, $P<0.01$.

MC3-R is not required for the inhibition of feeding by PYY_{3-36}

Peripherally administered PYY_{3-36} was reported to increase POMC neuronal firing in hypothalamic slices (Batterham et al., 2002) and POMC mRNA expression acutely (Challis et al., 2003). However, ip injection of PYY_{3-36} only increased the expression of c-Fos, an indirect marker of neuronal activation, in approximately 10-14% of ARC POMC neurons (Batterham et al., 2002). These results suggested to us that the role of the melanocortin system in mediating the anorexigenic actions of PYY_{3-36} needed to be examined more carefully. Indeed, in Chapter 1 we show that MC4-R is not required to mediate the anorexigenic actions of peripheral PYY_{3-36}. We further wanted to examine if peripheral PYY_{3-36} might be acting through the other central melanocortin receptor, MC3-R, to mediate is anorexigenic effects. MC3-$R^{+/+}$ (WT) and MC3-$R^{-/-}$ mice were acclimated to the procedure, and injected with PYY_{3-36} at 0.3, 3, and 10μg/100g after being fasted for 16 h, and their food intake measured every hour for 4 h. Peripheral PYY_{3-36} at 0.3, 3, and 10 μg/100g reduced food intake equally in MC3-$R^{+/+}$ and MC3-$R^{-/-}$ mice (Fig. 2). For example, at 3 μg/100g, PYY_{3-36} reduced food intake by 52 ±7% in MC3-$R^{+/+}$ and 42 ±11% in MC3-$R^{-/-}$ mice at 1 h (Fig. 2). This result indicates that MC3-R, like MC4-R, is not required to mediate the anorexigenic effects of peripheral PYY_{3-36}. Indeed, Agouti (Martin et al., 2004), and $POMC^{-/-}$ (Challis et al., 2004) mice all show, like MC3/4-$R^{-/-}$ mice, equal reduction of food intake in response to peripheral PYY_{3-36} administration, arguing that neither the POMC gene nor the melanocortin receptors are required for the anorexigenic actions of PYY_{3-36}.

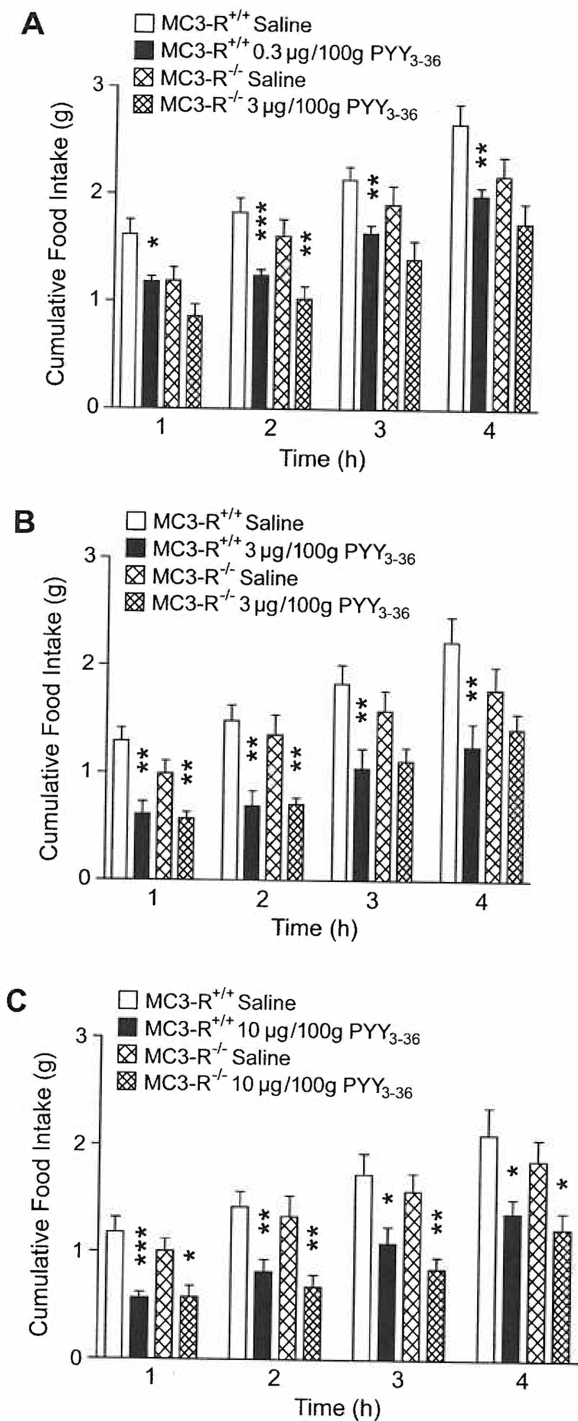


Fig. 2. MC3-$R^{+/+}$ and MC3-$R^{-/-}$ mice respond equivalently to increasing concentrations of PYY_{3-36} in a 16 h fast induced refeeding paradigm. A, 16 h fast induced refeeding response of MC3-$R^{+/+}$ and MC3-$R^{-/-}$ mice to a PYY_{3-36} dose of 0.3 μg / 100 g at 1, 2, 3, and 4 h post injection (n=9-10, each group). B, 16 h fast induced refeeding response of MC3-$R^{+/+}$ and MC3-$R^{-/-}$ mice to a PYY_{3-36} dose of 3 μg / 100 g at 1, 2, 3, and 4 h post injection (n=9-10, each group). C, 16 h fast induced refeeding response of MC3-$R^{+/+}$ and MC3-$R^{-/-}$ mice to a PYY_{3-36} dose of 10 μg / 100 g at 1, 2, 3, and 4 h post injection (n=9-10, each group). Data are expressed as mean ± SEM, statistics by two-way ANOVA followed by Student's (two-tailed) t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Corticotropin releasing factor receptors are not required for the anorexigenic actions of PYY_{3-36}

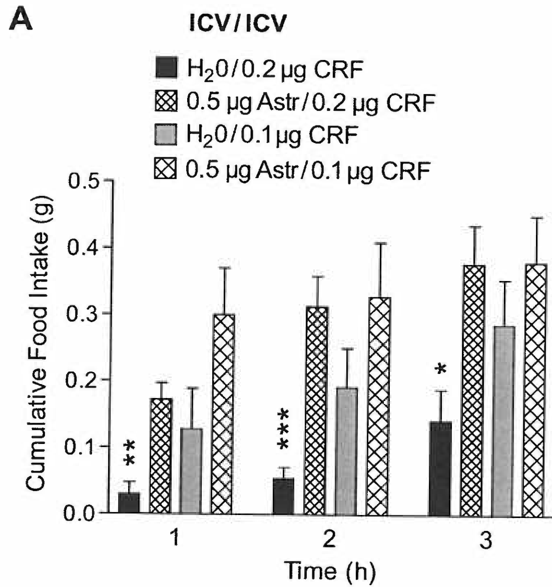
In Chapter 1 we showed that without proper habituation, WT mice do not respond to the anorexigenic effects of PYY_{3-36}. Furthermore, the stress caused by handling and injections, reduced food intake by 32% in unacclimated WT mice compared with acclimiated WT mice. This observation raised the possibility that stress-induced anorexia can mask the anorexigenic activity of PYY_{3-36}. Alternatively, PYY_{3-36} may be mediating its anorexigenic effects through stress-induced anorexia. Therefore, the inability of PYY_{3-36} to reduce food intake in a stressed state could be due to saturation of the system mediating its effects. Stress-induced anorexia is predominantly mediated trough the Corticotropin releasing factor (CRF) receptors, CRF_1 and CRF_2 receptors. With the use of specific agonists, CRF_1 receptors have been shown to have short-term inhibitory effects on feeding and stimulate colonic motility (Hotta et al., 1999; Tache et al., 2001). Additionally, peripheral and central CRF_2 receptors mediate the long-term inhibitory effects on feeding and reduce gastric acid secretion (Reyes et al., 2001; Wang et al., 2001). Peripheral PYY_{3-36} was originally described as "ilal or colonic break", acing peripherally and centrally to inhibit proximal gut motility and secretions, while increasing colonic motility (Introduction Section). To test the hypothesis that the anorexigenic effects of PYY_{3-36} are mediated by the CRF receptors, we use a non-specific CRF_1 and CRF_2 receptor antagonist, astressin, and tested if it can reverse the inhibition of food intake caused by PYY_{3-36}. We, initially, wanted to test the ability of astressin to block the CRF induced anorexia. In a 16 h fast induced refeeding paradigm, astressin (0.5 μg) was co-administered with either water or CRF (0.1 or 0.2 μg) intracerebroventricularly

(ICV) into the lateral ventricle at ~4:1, antagonist to agonist, ratio and food intake was measured hourly for 3 h (Fig. 3A). CRF dose dependently reduced food intake for the duration of the experiment (Fig. 3A). Administration of astressin 15 min prior to CRF (0.1 μg) injection, significantly reverse the reduction of food intake caused by CRF administration alone (Fig. 3A). This observation showed that astressin capable of reversing the anorexigenic activity of CRF receptors by being a non-selective CRF receptors competitive antagonist.

We next wanted to determine if the central CRF receptors are required for the anorexigenic effects of peripheral PYY_{3-36} (Fig. 3B). In a 16 h fast induced refeeding paradigm either water or astressin (0.5 μg) was administered ICV 15 min prior to peripheral PYY_{3-36} (5 μg/100g) (Fig. 3B). Although, a trend for stimulation of food intake with astressin administration was observed, compared to water treatment, it did not reach significance (Fig. 3B). Central astressin 15 min prior to peripheral PYY_{3-36} injection, has no effect on the reduction of food intake observed by peripheral PYY_{3-36} and central water treatment (Fig. 3B). At 1h, in mice that received a central water injection, PYY_{3-36} caused a 35 ±7% reduction of food intake, while in mice that received central astressin injection, it caused a 44 ±11% reduction (Fig. 3B). These results show that the central $CRF_{1/2}$ receptors are not essential for the action of PYY_{3-36}.

Like wise, peripheral administration of astressin at 30 μg/kg, a dose shown to block the effects of CRF receptors peripherally, had no effect on the reduction of food intake by ip PYY_{3-36} (5 μg/100g) in a 16 h fast induced refeeding paradigm (Fig. 4). At 2 h, PYY_{3-36} alone, reduced food intake by 45 ±7% and by 47 ±11% when astressin was co-

administered (Fig. 4). The results described above show that neither the peripheral nor the central $CRF_{1/2}$ receptors are essential for the action of PYY_{3-36}.



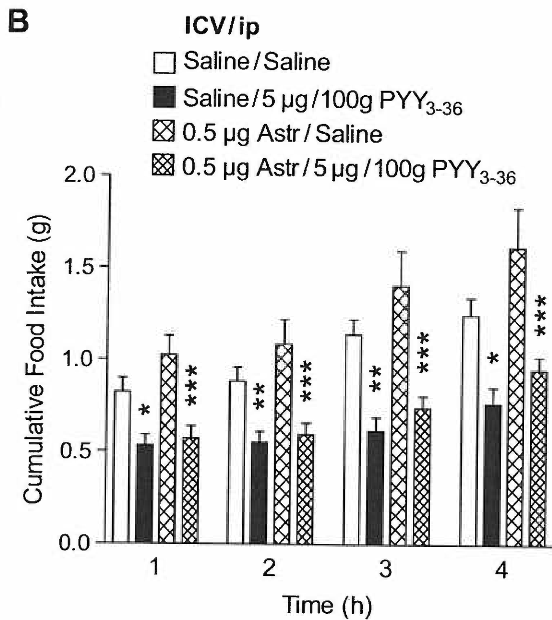


Fig. 3. Central intracerebroventricular (ICV) administration of astressin, a $CRF_{1/2}$ receptors antagonist, does not reverse the anorexigenic effects of peripheral PYY_{3-36}. in a 16 h fast induced refeeding paradigm. Mice were stereotaxically implanted with cannulas using standard procedures as described in (Fan et al., 2000). In short, mice were anesthetized with isoflorine and a sterile guide cannula was stereotaxically (CARTESIAN Research, Inc., Sandy, OR) implanted into the right lateral ventricle (0.5 mm relative to bregma, 1 mm lateral to midline, and 2-2.2 mm below the surface of the skull). The cannulla was then fixed in place using dental cement. The animals were individually housed for a week after surgery and acclimated to experiments (as described in Methods of Chapter 1). The positions of the cannulae were verified by injecting a dye.
A, ICV administration of astressin (0.5 µg), 15 min prior to CRF (0.1 or 0.2 µg), reverses the anorexigenic effects of ICV CRF injection (n=8-10). B, ICV administration of astressin (0.5 µg), 15 min prior to ip PYY_{3-36} (0.5 µg/100g), does not affect the reduction of food intake by PYY_{3-36} (n=8-10). Data are expressed as mean ± SEM, statistics by two-way ANOVA followed by Student's (two-tailed) t-test: *, $P<0.05$; **, $P<0.01$; ***,$P<0.001$.

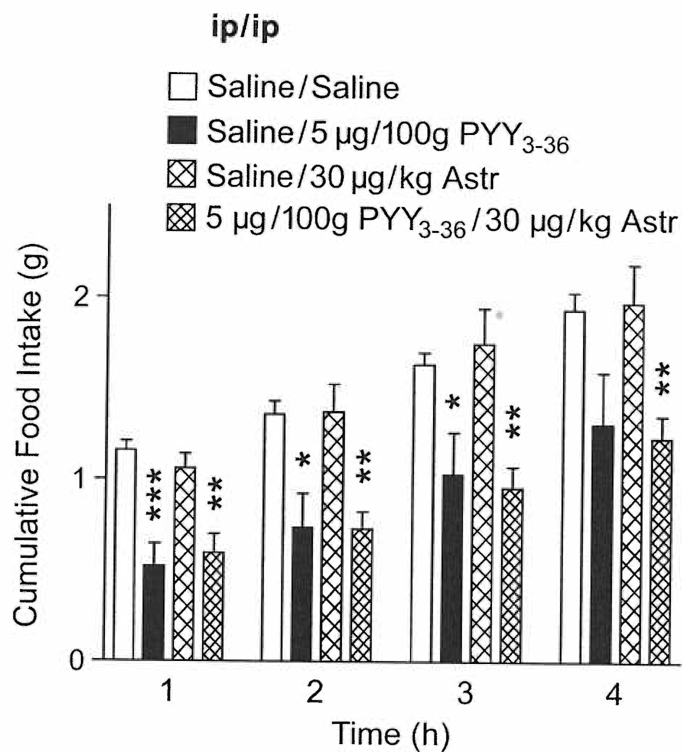


Fig. 4. Peripheral co-administration of astressin, a $CRF_{1/2}$ receptors antagonist, dose not affect on the anorexigenic actions of ip PYY_{3-36} (5 µg/100g) (n=6-8). Data are expressed as mean ± SEM, statistics by two-way ANOVA followed by Student's (two-tailed) t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Peripheral $PYY_{3\text{–}36}$ does not induce c-Fos, a marker of neuronal activation, in tyrosine hydroxylase (TH) NTS neurons

The initial report by Batterham and coworkers hypothesized that $PYY_{3\text{–}36}$ mediates its anorexigenic effect through the ARC of the hypothalamus (Batterham et al., 2002). However, two reports have raised the possibility that the ARC nucleus may not be the primary site of action of peripheral $PYY_{3\text{–}36}$. In one, peripheral administration of $PYY_{3\text{–}36}$ caused taste aversion in mice and a dose dependent activation of c-Fos in AP and intermediate nucleus tractus solitarius (iNTS) (Halatchev and Cone, 2005). The iNTS and AP are areas shown to respond to aversion (Schafe et al., 1995). In the second, vagotomy abolished the induction of ARC c-Fos by peripherally administered $PYY_{3\text{–}36}$ (Koda et al., 2005) in rats. Finally, in Chapter 3 we showed that peripherally administered $PYY_{3\text{–}36}$ inhibits food intake and activates c-Fos in the iNTS of ARC leasioned MSG-treated mice, suggesting that the iNTS may be mediating its anorexigenic actions. We, therefore, wanted to determine the neurochemical identity of the iNTS neurons activated by peripheral $PYY_{3\text{–}36}$. We hypothesized that $PYY_{3\text{–}36}$ may mediate part of its anorexigenic affect through the TH, sympathetic, neurons in the NTS because of the neuroanatomical position of c-Fos activation and NTS TH neurons are activated by other gut peptide, like CCK (Monnikes et al., 1997). Nocturnal ip injection (1900 h) of $PYY_{3\text{–}36}$ (5 μg/100g) (Methods in Chapter 3) did not induce c-Fos activation in NTS TH neurons compared to a saline treatment (Fig. 5). The total number of TH positive neurons was the same in saline and ip $PYY_{3\text{–}36}$ treated mice (Fig. 5A). Peripheral administration of $PYY_{3\text{–}36}$ did not increase the number of TH neurons expressing c-Fos (Fig. 5C), even though, it caused a significant induction of c-Fos in NTS section

containing TH neurons (Fig. 5B). This observation indicates that the anorexigenic and aversive effects of peripheral PYY_{3-36} might not be mediated NTS TH neurons.

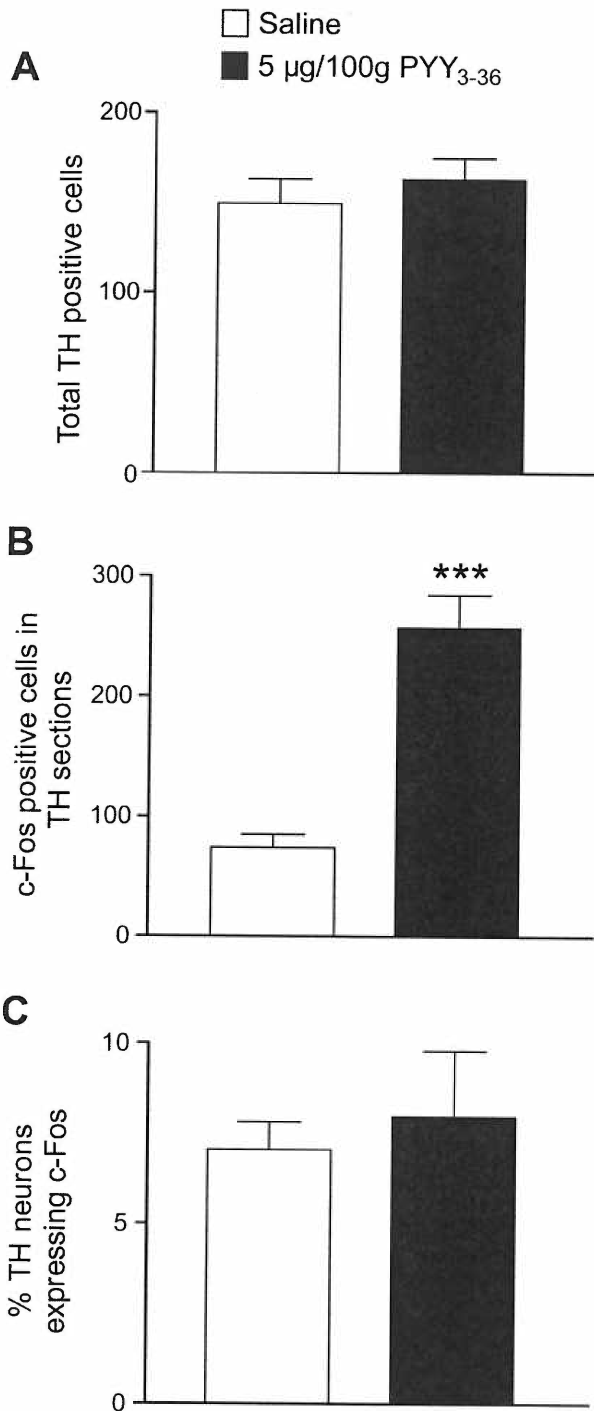


Fig. 5. Peripheral PYY_{3-36} does not activate c-Fos expression in NTS TH neurons. A, Total TH positive cell in the NTS' of saline and PYY_{3-36} (5 μg/100g) treated mice. B, Peripheral PYY_{3-36} (5 μg/100g) causes a 3-fold increase in c-Fos in NTS sections positive for TH, compared to saline treatment. C, There is no significant increase in c-Fos expression in NTS TH neurons after ip administration of PYY_{3-36} (5 μg/100g) (n=4). Data are expressed as mean ± SEM, statistics by Student's (two-tailed) t-test: ***,$P<0.001$.

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