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Hypothalamic melanocortin neurons integrate signals of energy state

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Abstract

Neurons of the arcuate nucleus of the hypothalamus appear to be sites of convergence of central and peripheral signals of energy stores, and profoundly modulate activity of the melanocortin circuits, providing strong rationale for pursuing these circuits as therapeutic targets for disorders of energy homeostasis. Recent studies in our lab and those of our collaborators have shown that leptin modulates different populations of hypothalamic cells in different ways. In this report, we outline an integrated model of leptin's action in the arcuate nucleus, derived from our electrophysiological studies of brain slice preparations taken from transgenic mice bred to express a variety of fluorescent proteins in specific cell types. We also discuss the recently withdrawn obesity drug fenfluramine, which appears to act on proopiomelanocortin neurons via serotonin $_{2C}$ receptors. Finally, we review current inquiries into the ability of the hormone ghrelin to stimulate appetite by its activation of neuropeptide Y neurons and inhibition of proopiomelanocortin neurons.

Keywords: Leptin; Proopiomelanocortin; Agouti-related peptide; Neuropeptide Y; Ghrelin; Peptide YY-(3–36)

1. Introduction

Obesity is one of the major health challenges facing the developed world, accounting for 280,000 deaths annually in the USA (Peeters et al., 2003). Being obese or overweight decreases life expectancy between 3 and 13 years (Fontaine et al., 2003; Peeters et al., 2003), and obesity significantly increases the risk of coronary disease, diabetes and some types of cancer (Bergstrom et al., 2001). There are also significant economic costs associated with health care for such an obese population. For example, an estimated 9.4% of US healthcare expenditure is directly related to "obesity and inactivity," while recent costs due to diabetes were estimated at US$98 billion per annum (Mokdad et al., 2001). In general, European demographic statistics on obesity and inactivity have shown a trend of increasing incidence in both children and adults, as living standards have improved over the past four decades (Micic, 2001; Moreno et al., 2002).

Early studies identified the hypothalamus as an important center of energy homeostatic control (Hetherington and Ranson, 1940) and recently the role of the arcuate nucleus of the hypothalamus has been highlighted (Cone et al., 2001). There have also been tremendous advances in identifying genes and pathways important for regulating energy homeostasis (Spiegelman and Flier, 2001), in particular the hormone–receptor pair leptin (*Lep*^{ob}) (Zhang et al., 1994) and leptin receptor (*Lepr* or LR) (Tartaglia et al., 1995). White adipose cells produce leptin (Zhang et al., 1994) and the absence of leptin or functional leptin receptors causes morbid obesity (Spiegelman and Flier, 2001). The "long" leptin receptor b has a 301 amino acid intracellular tail (Chua et al., 1996, 1997; Tartaglia et al., 1995) and is expressed at high levels in so-called "satiety" centers in the hypothalamus as well as in some non-neuronal tissues, such as pancreatic β cells (Elmquist et al., 1998a,b; Emilsson et al., 1997; Fei et al., 1997; Tartaglia et al., 1995). A point mutation in the leptin receptor b splice junction results in the premature truncation of the receptor in *db* mice (Chen et al., 1996; Chua et al., 1996; Lee et al., 1996; Tartaglia et al., 1995). Thus, while the role of the short leptin receptor forms remains unclear, leptin receptor b function is critical for maintenance of endocrine function and energy balance.

2. Neurons in the arcuate nucleus are a major target of peripheral leptin

Leptin receptor b is expressed highly in the hypothalamus, in the arcuate nucleus of the hypothalamus, in the

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ventromedial hypothalamic nucleus, and in the dorsomedial hypothalamic nucleus, with lower expression in the paraventricular hypothalamic nucleus, and the lateral hypothalamus (Elmquist et al., 1998a; Fei et al., 1997; Mercer et al., 1996b; Schwartz et al., 1996). The value of immunostaining for leptin receptor b remains controversial but in situ hybridization histochemical studies have unequivocally demonstrated that that the fore-mentioned regions express leptin receptor b. These regions have all been implicated in the control of energy homeostasis (Elmquist et al., 1998b). One of the major targets for circulating leptin is the arcuate nucleus. Leptin receptors were first identified on cells in the arcuate nucleus and ventromedial hypothalamic nucleus (Mercer et al., 1996a) and radiolabeled leptin binds in the arcuate nucleus after peripheral injection (Banks et al., 1996). Furthermore, peripheral injection of leptin stimulates robust expression of the immediate early gene c-*fos* (a marker of neuronal activity) and expression of the leptin marker SOCS3 (suppressor of cytokine signallling-3) (Bjorbaek et al., 1998) in neurons within this nucleus (Elias et al., 1999). The permeability of the blood–brain barrier in this region (Banks et al., 1996) means that circulating leptin has better access to leptin receptors here. Leptin is specifically transported across the blood–brain barrier (Banks et al., 1996) to reach sites distal to the circumventricular organs, and saturation of these transporters is hypothesized to be part of the cause of diet-induced obesity. Leptin exerts some of its effects on energy homeostasis by complementary actions on both neuropeptide Y/agouti-related peptide and proopiomelanocortin neurons, and both these neuron types express leptin receptors (Cheung et al., 1997; Hakansson et al., 1996).

3. Genomic actions of leptin on neuropeptide Y/agouti-related peptide and proopiomelanocortin neurons

Neuropeptide Y has well-described effects on energy homeostasis (see Kalra et al., 1999 for review). Neuropeptide Y is produced in hypothalamic nuclei known to regulate appetite and metabolism, and neuropeptide Y is one of the most potent orexigens. Intracerebroventricular injection of neuropeptide Y will profoundly stimulate feeding (Billington et al., 1991; Stanley et al., 1985) and will reduce energy expenditure (Billington et al., 1991). It has been demonstrated that leptin modulates neuropeptide Y levels; leptin deficiency results in a significant increase in neuropeptide Y mRNA expression while leptin administration causes a decrease in expression (Stephens et al., 1995). Furthermore, genetic deletion of neuropeptide Y in leptin-deficient animals resulted in a significant decrease in their degree of obesity (Erickson et al., 1996). Treatment of Lep^{ob}/Lep^{ob} mice with leptin reduced their hyperphagia and normalized neuropeptide Y expression, while neuropeptide Y synthesis and secretion are increased in most models of energy deficiency or increased metabolic demand (Inui, 1999). Interestingly, neuropeptide Y neurons co-express agouti-related peptide (Broberger et al., 1998), a potent antagonist of the melanocortin 3 and 4 receptors (MC_3 receptor and MC_4 receptor). Agouti-related peptide expression is robustly stimulated by fasting and inhibited by leptin (Broberger et al., 1998; Mizuno and Mobbs, 1999; Shutter et al., 1997), and leptin-deficient mice have elevated agouti-related peptide mRNA levels (Mizuno and Mobbs, 1999; Shutter et al., 1997).

Leptin modulates proopiomelanocortin mRNA (Thornton et al., 1997) (although to a much lower extent than leptin modulates agouti-related peptide mRNA) and leptin-deficient mice have reduced levels of proopiomelanocortin transcript (Wilding et al., 1993). The cloning of the agouti gene (Bultman et al., 1992) and the discovery of the mechanism of agouti's action (Fan et al., 1997; Lu et al., 1994) led to the proposal that the melanocortin system, specifically the proopiomelanocortin neurons acting via α-melanocyte stimulating hormone and the MC_4 receptor, acts centrally to limit adipose stores and to stimulate energy expenditure. This hypothesis was supported by the demonstration of hyperphagia and obesity in MC_4 receptor-deficient mice (Huszar et al., 1997), by the characterization of agouti-related peptide as an endogenous hypothalamic melanocortin receptor antagonist (Fong et al., 1997; Ollmann et al., 1997), and by the obesity evident in transgenic mice that ectopically over-express agouti-related peptide (Ollmann et al., 1997).

Thus, it appears that leptin inhibits the expression of orexigenic neuropeptides and increases the expression of anorexigenic neuropeptides. However, it is important to remember that the brain is not a "neuropeptide soup," and that neuropeptides only modulate neurotransmission. To better understand how leptin acts on the brain, it is necessary to understand the effects of leptin on neurotransmission and neuronal activity.

4. Leptin rapidly activates some hypothalamic neurons

Soon after its discovery, leptin was shown to cause an increase in the expression of immunoreactive c-*fos* not only in the medio-basal hypothalamus, especially the paraventricular hypothalamic nucleus, but also in the arcuate nucleus and the ventromedial hypothalamic nucleus of Lep^{ob}/Lep^{ob} mice (Woods and Stock, 1996). This suggested an increase in the activity of neurons in these regions, although the neuropeptide identity of the activated cells was not determined. This work was further refined when it was shown that peripheral injection of leptin rapidly induced c-*fos* expression in proopiomelanocortin neurons, while it did not induce c-*fos* in neuropeptide Y neurons (Elias et al., 1999). Both proopiomelanocortin and neuropeptide Y neurons showed increased expression of mRNA for suppressor of cytokine signaling-3 (SOCS3). The authors proposed that leptin was acting differentially on the two neuronal popu-

lations, to activate proopiomelanocortin neurons and to inhibit the neuropeptide Y/agouti-related peptide neurons. Leptin rapidly stimulates the secretion of α-melanocyte stimulating hormone from a hypothalamic slice preparation (Kim et al., 2000; Watanobe and Habu, 2002). Leptin can also depolarize parvocellular paraventricular hypothalamic neurons (Powis et al., 1998), through activation of a depolarizing, nonspecific cation current, and leptin has been shown to have effects on sympathetic outflow within 15 min (Haynes et al., 1997), demonstrating rapid (non-genomic) effects of leptin.

5. Leptin rapidly inhibits some hypothalamic neurons

In whole-cell electrophysiological recordings, leptin was shown to inhibit the activity of some neurons in the arcuate nucleus (Glaum et al., 1996) and to hyperpolarize and decrease the action potential firing rate of unidentified arcuate nucleus and ventromedial hypothalamic neurons (Spanswick et al., 1997). The inhibitory effect on arcuate nucleus and ventromedial hypothalamic neurons was due to activation of an adenosine 5′ -triphosphate-sensitive potassium channel (K_{ATP}). The consequent efflux of K^+ ions from the cell reduced the membrane potential and this hyperpolarization led to less frequent action potentials. These responses were absent in Zucker fatty rats ($Lepr^{fa}/Lepr^{fa}$) (Glaum et al., 1996; Spanswick et al., 1997), which lack functional leptin receptors.

Interestingly, the neurons that were hyperpolarized by leptin were also hyperpolarized by glucose, also acting through the K_{ATP} channel. The convergence of glucose and leptin signals onto hypothalamic neurons has been confirmed (Shiraishi et al., 1999). In particular, it appears that modulation of the K_{ATP} channel is a common mechanism used by glucose, insulin, and leptin (Harvey and Ashford, 1998a,b,c; Harvey et al., 1997, 2000; Levin et al., 1999, 2001; Spanswick et al., 1997, 2000) to change cellular activity.

In some circumstances, leptin can rapidly inhibit secretion of neuropeptide Y and agouti-related peptide from hypothalamic slices (Li et al., 2000). Surprisingly, other investigators have not been able to replicate this effect in hypothalamic explants (King et al., 1999) or in vivo (Watanobe and Habu, 2002); the reasons for this discrepancy are not clear.

6. Integrated model of leptin action in the arcuate nucleus of the hypothalamus

Thus, leptin may exert diverse effects on neuronal activity depending upon the neuropeptide phenotype of the neuron that is responding. To understand leptin actions on the brain, it is necessary to know the neuropeptide phenotype of the leptin-sensitive neurons. In a recent publication (Cowley et al., 2001), we have offered a model by which leptin directly and differentially acts on neurons in the arcuate nucleus. We formulated the model of arcuate nucleus circuitry based on electrophysiological data we generated by recordings from identified proopiomelanocortin neurons in brain slices taken from transgenic mice. Our collaborators, Malcolm Low and Jim Smart, developed a transgenic mouse termed the proopiomelanocortin–enhanced green fluorescent protein (POMC–EGFP) mouse; these mice express a transgene incorporating 11 kb of the proopiomelanocortin gene promoter and a gene for enhanced green fluorescent protein (Fig. 1A) to cause eutopic expression of enhanced green fluorescent protein in proopiomelanocortin neurons.

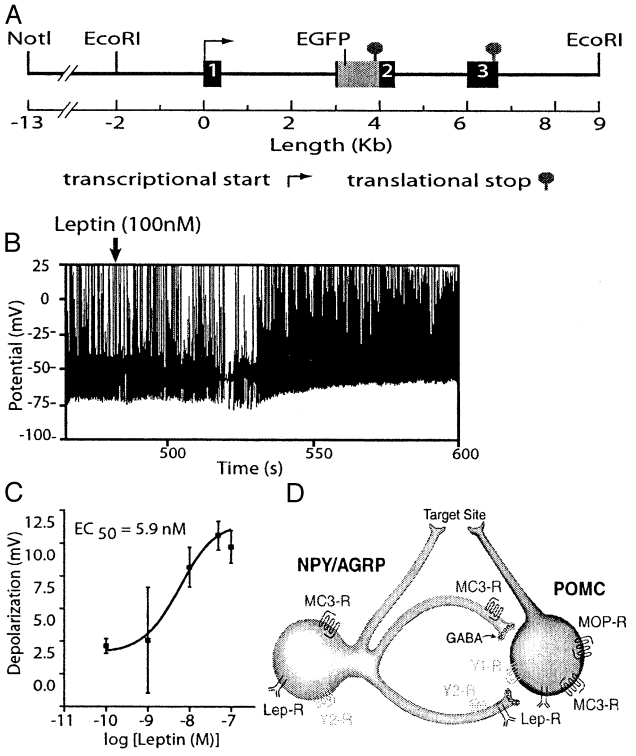

Fig. 1. Proopiomelanocortin neurons are activated by leptin. (A) Structure of the proopiomelanocortin–enhanced green fluorescent protein (POMC–EGFP) transgene. (B) Leptin depolarizes proopiomelanocortin neurons and increases the frequency of action potentials within 1 to 10 min of addition. The figure is a representative example of recordings made from 77 proopiomelanocortin neurons. (C) Leptin causes a concentration-dependent depolarization of proopiomelanocortin cells. The depolarization caused by leptin was determined at 0.1, 1, 10, 50, and 100 nM (EC_{50} = 5.9 nM) in (8, 7, 9, 3, 45) cells, respectively. (D) Model of leptin regulation of neuropeptide Y/gamma-amino-butyric acid and proopiomelanocortin neurons in the arcuate nucleus of hypothalamus. Leptin directly depolarizes the proopiomelanocortin neurons, simultaneously hyperpolarizes the somata of neuropeptide Y/GABA neurons, and diminishes release from neuropeptide Y/GABA terminals. This diminished GABA release disinhibits the proopiomelanocortin neurons. Together, the direct and indirect effects of leptin result in an activation of proopiomelanocortin neurons and an increased frequency of action potentials (adapted from Cowley et al., 2001), with permission.

To determine the effects of specific hormones on the activity of proopiomelanocortin neurons, we cut coronal sections of the brains of POMC–EGFP transgenic mice and performed standard electrophysiological recordings from neurons that showed enhanced green fluorescent protein fluorescence. Application of leptin to the tissue bath depolarized proopiomelanocortin neurons (Fig. 1B) from a resting potential of −40 to −45 mV in a concentration-responsive manner (Fig. 1C).

Two components contributed to the depolarization of the proopiomelanocortin neurons: Leptin increased the conductance of a nonspecific cation channel on proopiomelanocortin neurons, an effect that other investigators had seen with leptin before (Powis et al., 1998), and leptin decreased the frequency of inhibitory currents onto proopiomelanocortin neurons. In studies with Tamas Horvath, we showed that the majority of the gamma-aminobutyric acid (GABA)-ergic inputs to proopiomelanocortin neurons also expressed neuropeptide Y, suggesting that there was a local circuit within the arcuate nucleus—that neuropeptide Y neurons innervated and provided a dominant inhibitory tone onto proopiomelanocortin neurons. We also showed that leptin inhibited the release of GABA and neuropeptide Y from neuropeptide Y terminals in the arcuate nucleus. Thus, leptin was having different actions on different classes of neurons and the net effect was to increase the tone of anorectic proopiomelanocortin neurons, and decrease the tone of the orexigenic neuropeptide Y/agouti-related peptide neurons. We then went on to test whether this model circuit (Fig. 1D) was sensitive to other signals that regulate energy homeostasis.

7. Fenfluramine action shows how serotonin affects energy homeostasis

Before its withdrawal from the market because of cardiac complications in a subset of patients, fenfluramine was the most successful pharmacotherapy for obesity. Fenfluramine increased the concentration of serotonin (5-HT) at synapses, but the mechanism by which it caused weight loss was unknown. We investigated the effect of fenfluramine on proopiomelanocortin neurons and other compounds that activate the serotonergic system, as part of an effort to determine how fenfluramine caused weight loss.

We showed that fenfluramine increased the firing rate of proopiomelanocortin neurons (Fig. 2), and that this was due to a depolarization of the neurons via activation of the serotonin 2C receptor (5-HT_{2C} receptor). This was in accordance with functional anatomical and physiological data generated by our collaborators in Joel Elmquist's laboratory, showing that fenfluramine increased the expression of c-*fos* in proopiomelanocortin neurons that expressed the 5-HT_{2C} receptor, and that fenfluramine required a functional melanocortin system to inhibit

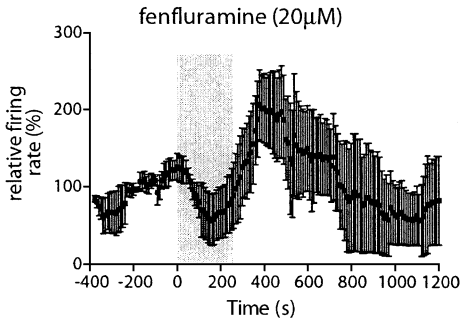

Fig. 2. Fenfluramine increases the activity of proopiomelanocortin neurons. Fenfluramine (20 μM) caused a two-fold increase in the spontaneous action potential frequency of proopiomelanocortin neurons from POMC–EGFP mice when recorded in the loose-cell-attached mode (adapted from Heisler et al., 2002), with permission.

feeding. These combined findings led us to deduce that fenfluramine acted through 5-HT_{2C} receptors and the melanocortin system (Heisler et al., 2002) to inhibit feeding, and ultimately to reduce body weight. We hope that an understanding of the role of the 5-HT_{2C} receptor in control of feeding and energy expenditure will lead to better therapies for obesity.

These findings also suggest that nutrient-sensing serotonin neurons may project from the raphe nuclei in the brainstem to the hypothalamus. Within the arcuate nucleus of the hypothalamus, serotonin signals are integrated with other signals like leptin, and as will be shown later, ghrelin and peptide YY-(3–36) from the gut, to produce a coordinated response to nutrient state. This signal is likely propagated through the brain by the coordinated effects of neuropeptide Y, agouti-related peptide, and α-melanocortin-stimulating hormone (Cowley et al., 1999).

8. Neuropeptide Y-sensitive GABA release highlights a new role for peptide YY-(3–36)

To establish the identity of the GABAergic inputs to proopiomelanocortin neurons, we analyzed the neuropeptide Y sensitivity of the GABA release. We reasoned that a subtype of the neuropeptide Y receptor, the Y2 receptor (NPY Y_2 receptor) was expressed on neuropeptide Y neurons in the arcuate nucleus and may act as an autoreceptor to limit the activity of neuropeptide Y neurons when the local concentrations of neuropeptide Y rise too high. We found that the GABA release onto proopiomelanocortin neurons was extraordinarily sensitive to neuropeptide Y, and later to neuropeptide Y Y_2 receptor-specific agonists. This led us to propose that neuropeptide Y Y_2 receptor agonists would decrease the activity of neuropeptide Y/agouti-related peptide neurons, and thus increase the activity of proopiomelanocortin neurons, causing similar effects on feeding and energy homeostasis to leptin. Because the arcuate nucleus blood–brain barrier is more permeable than in other parts

of the brain, we reasoned that circulating hormones might have access to these Y_2 receptor sites.

One candidate ligand to act on the neuropeptide Y Y_2 receptor is peptide YY-(3–36) (PYY-(3–36)). It had long been known that peptide YY-(3–36) was released after a meal, but its function was not clear. We showed that peptide YY-(3–36) decreased the frequency of inhibitory post-synaptic currents (IPSCs) due to GABA release, onto proopiomelanocortin neurons (Fig. 3A), an effect we have previously shown to be due to activity of neuropeptide Y neurons, indicating that peptide YY-(3–36) inhibited neuropeptide Y neurons. We also showed that peptide YY-(3–36) activated proopiomelanocortin neurons (Fig. 3B), increasing the neurons' spontaneous activity. In collaboration with us, Rachel Batterham in the Bloom laboratory showed that the effect of this dual inhibition of neuropeptide Y neurons and activation of proopiomelanocortin neurons was that Y_2 receptor agonists and peptide YY-(3–36) inhibit feeding in rats and mice. Furthermore, in studies with Herbert Herzog it was shown

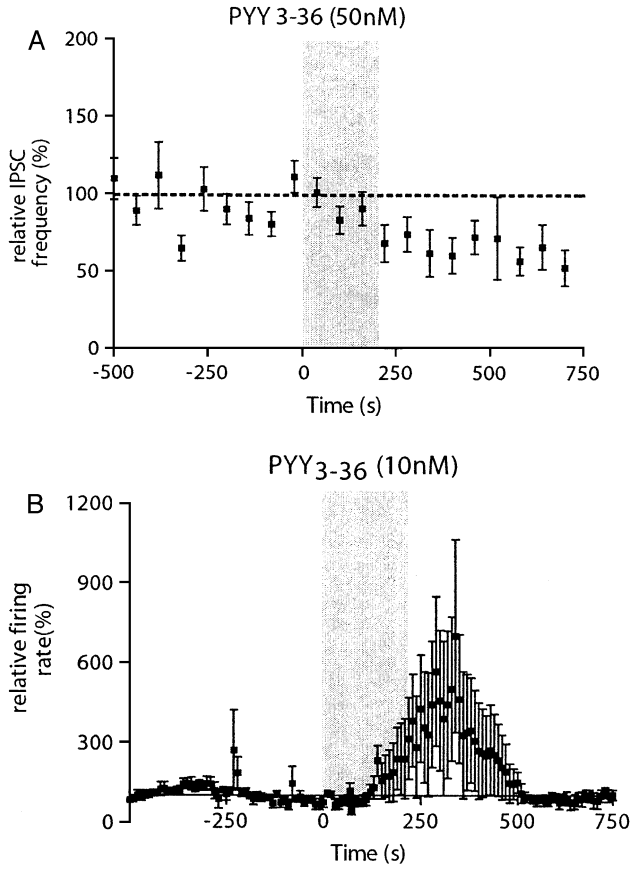

Fig. 3. The gut hormone peptide YY-(3–36) inhibits neuropeptide Y neurons and thus activates proopiomelanocortin neurons. (A) Peptide YY-(3–36) (50 nM) decreased the frequency of spontaneous mini-inhibitory post-synaptic currents (IPSCs) onto proopiomelanocortin neurons, when recorded in voltage clamp configuration. (B) Peptide YY-(3–36) (10 nM) caused a four-fold increase in the frequency of spontaneous action potentials in proopiomelanocortin neurons from POMC–EGFP mice when recorded in the loose-cell-attached configuration (adapted from Batterham et al., 2002), with permission.

that peptide YY-(3–36) had no effect on neuropeptide Y Y_2 receptor-deficient mice. It was also shown that infusion of post-prandial concentrations of peptide YY-(3–36) caused reduced appetite and food consumption in human volunteers (Batterham et al., 2002). These findings have led to renewed interest in peptide YY-(3–36) and related compounds as possible therapies for obesity. Strategies that activate these neurons in a similar manner to, but independently of leptin, may bypass the resistance to leptin seen in most human obesity. These findings are also an example of the predictive power of the melanocortin circuitry model we have devised. Observations about the nature of the circuitry led us to hypothesize and demonstrate a new gut-brain axis that limits food intake.

9. Ghrelin acts opposite to peptide YY-(3–36) on melanocortin circuits

The findings reported so far all show how hormones can inhibit food intake and increase energy expenditure by activating proopiomelanocortin neurons and stimulating the melanocortin system. It is reasonable to wonder whether this system can be modified in the other direction—that is, can hormones stimulate feeding by inhibiting the melanocortin system? There is more than an academic interest in this, because cachexia and wasting disorders are significant medical problems (Marks et al., 2001).

Ghrelin is a hormone that is secreted by A-like cells of the stomach (Dornonville de la Cour et al., 2001), it stimulates growth hormone secretion, and independently stimulates appetite (Cummings et al., 2001; Kojima et al., 1999). There is also evidence that ghrelin may be an important factor in the long-term success of gastric bypass procedures for weight control (Cummings et al., 2002). Previous data suggest that ghrelin acts in the arcuate nucleus of the hypothalamus; however, the mechanism of that effect is unclear. We sought to determine the mechanism by which ghrelin stimulates feeding. In a recent publication, we demonstrate that ghrelin activates neuropeptide Y neurons and causes a secondary inhibition of proopiomelanocortin neurons (Cowley et al., 2003b).

To determine the mechanism of ghrelin action on hypothalamic neuronal activity, we used two models: The POMC–EGFP mouse described above and another transgenic mouse, which expresses sapphire fluorescent protein under the control of the neuropeptide Y promoter. The neuropeptide Y-sapphire fluorescent protein mouse was developed by our collaborators in the Friedman laboratory and directs eutopic expression of sapphire fluorescent protein to neuropeptide Y neurons, including arcuate nucleus neuropeptide Y neurons.

Bath application of ghrelin increased the activity of arcuate nucleus neuropeptide Y neurons (Fig. 4A), stimulating the release of GABA, neuropeptide Y and agouti-related peptide. The coordinated actions of these signals hyper-

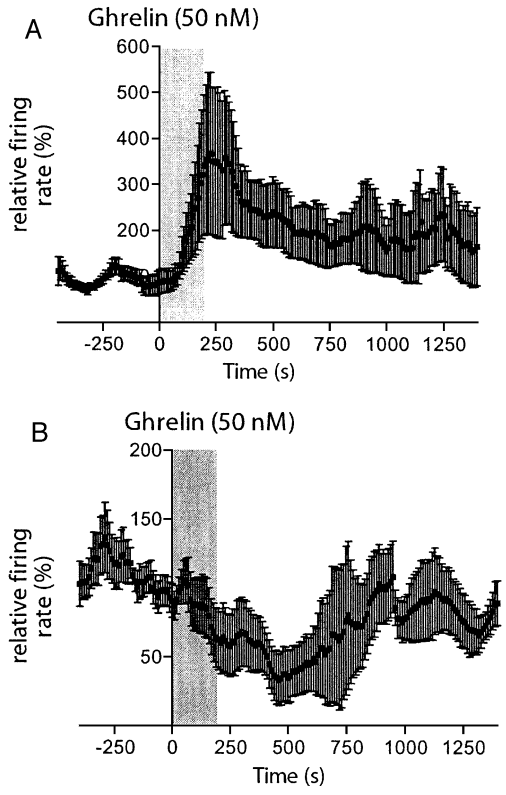

Fig. 4. The stomach hormone ghrelin activates neuropeptide Y neurons and thus inhibits proopiomelanocortin neurons. (A) Ghrelin (50 nM) caused a four-fold increase in the spontaneous action potential frequency of neuropeptide Y neurons, measured using loose-cell-attached patch recordings. (B) Ghrelin (50 nM) caused a 50% decrease in the spontaneous activity of proopiomelanocortin neurons, using loose-cell attached patch recordings (from Cowley et al., 2003b), with permission.

polarized and reduced the spontaneous activity of the proopiomelanocortin neurons (Fig. 4B). The effect of ghrelin on proopiomelanocortin neurons was secondary to the effect on neuropeptide Y neurons; in this manner ghrelin is acting like peptide YY-(3–36) but causing the opposite effects.

Our collaborators in the Horvath and Smith laboratories went on to identify a population of neurons in the hypothalamus that express ghrelin and send fibers to contact neuropeptide Y neurons in many regions of the hypothalamus, and Heiman et al. showed that ghrelin binds to neuropeptide Y neurons. Ghrelin fibers also make contacts with other kinds of hypothalamic neurons. In the same publication, the Colmers laboratory showed that ghrelin caused a decreased release of GABA onto corticotrophin releasing hormone neurons and that this effect required neuropeptide Y actions. This suggests that ghrelin was stimulating the release of neuropeptide Y adjacent to corticotrophin releasing hormone neurons, and we have previously shown that neuropeptide Y reduces GABAergic inhibitory post-synaptic currents (Cowley et al., 1999) in the paraventricular hypothalamus. Furthermore, this finding provides a potential mechanism for the previously demonstrated stimulation of adrenocorticotrophic hormone by ghrelin (Arvat et al., 2001).

10. Conclusion

These data show that arcuate nucleus neurons are sites of convergence of central and peripheral signals of energy stores (Fig. 5). Signals from the body and the brain

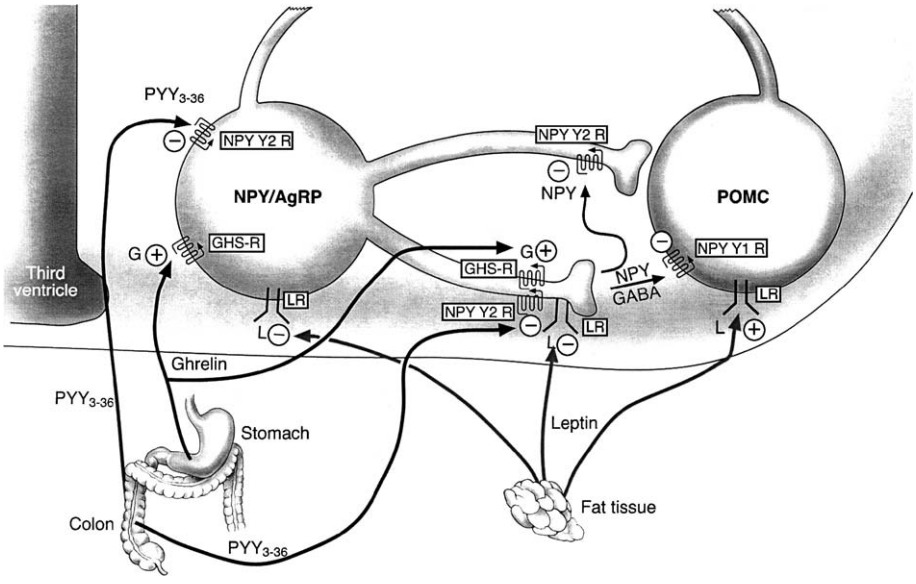

Fig. 5. A model of convergence and action of peripheral hormones on melanocortin circuits in the arcuate nucleus of the hypothalamus (from Cowley et al., 2003a), with permission.

converge on the melanocortin circuits here and profoundly modulate the activity of those circuits. The new findings reviewed here also point to the importance of the inhibitory tone of the neuropeptide Y/GABA synapses onto proopiomelanocortin neurons. Both the gut hormones characterized here act on the neuropeptide Y neurons and cause a reciprocal change in the activity of the proopiomelanocortin neurons. The data described here also provide strong rationale for pursuing melanocortin circuits as therapeutic targets for disorders of energy homeostasis.

Acknowledgements

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