The Distribution and Mechanism of Action of Ghrelin in the CNS Demonstrates a Novel Hypothalamic Circuit Regulating Energy Homeostasis

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Summary

The gastrointestinal peptide hormone ghrelin stimulates appetite in rodents and humans via hypothalamic actions. We discovered expression of ghrelin in a previously uncharacterized group of neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei. These neurons send efferents onto key hypothalamic circuits, including those producing neuropeptide Y (NPY), Agouti-related protein (AGRP), proopiomelanocortin (POMC) products, and corticotropin-releasing hormone (CRH). Within the hypothalamus, ghrelin bound mostly on presynaptic terminals of NPY neurons. Using electrophysiological recordings, we found that ghrelin stimulated the activity of arcuate NPY neurons and mimicked the effect of NPY in the paraventricular nucleus of the hypothalamus (PVH). We propose that at these sites, release of ghrelin may stimulate the release of orexigenic peptides and neurotransmitters, thus representing a novel regulatory circuit controlling energy homeostasis.

Introduction

Ghrelin was discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R); it is predominantly produced in the stomach and potently stimulates growth hormone (GH) secretion (Kojima et al., 1999). The octanoylated peptide hormone is produced and secreted by A-like cells (Dornonville de la Cour et al., 2001) within the oxyntic glands of the stomach (Sakata et al., 2002). The testis (Tanaka et al., 2001), placenta (Gualillo et al., 2001), kidney (Mori et al., 2000), pituitary (Korbonits et al., 2001), small intestine (Date et al., 2000), pancreas (Volante et al., 2002), lymphocytes (Hattori et al., 2001), and brain (Lu et al., 2001) also express low levels of ghrelin. Ghrelin peptide secretion and mRNA expression is increased by weight loss, restriction of caloric intake (Tschop et al., 2000; Cummings et al., 2001), and insulin-induced hypoglycemia (Toshinai et al., 2001). Caloric intake (Tschop et al., 2001a) or chronically positive energy balance (Tschop et al., 2001b) suppress ghrelin expression and secretion. This suggests that ghrelin may act as a signal of nutrient status directly from the gut (Horvath et al., 2001). Ghrelin stimulates GH secretion (Kojima et al., 1999; Arvat et al., 2000), increases food intake, and decreases fat utilization. Importantly, the weight gain and adiposity caused by such positive energy balance is independent of ghrelin's ability to modulate GH secretion (Tschop et al., 2000; Wren et al., 2000; Nakazato et al., 2001). Ghrelin increases the expression of mRNA for AGRP and NPY and triggers the expression of immediate early genes in the medial arcuate nucleus, an area rich in NPY/AGRP neurons (Dickson and Luckman, 1997; Hewson and Dickson, 2000; Wang et al., 2002). The influence of ghrelin on NPY and AGRP pathways in this area appears to be complementing the actions of leptin by acting in

A

320bp ►

13KDa ►

Hyp   Stom

B

PVH
f
AHA
-1.88 mm

LH
VMH
Arc
-2.30 mm

DMH
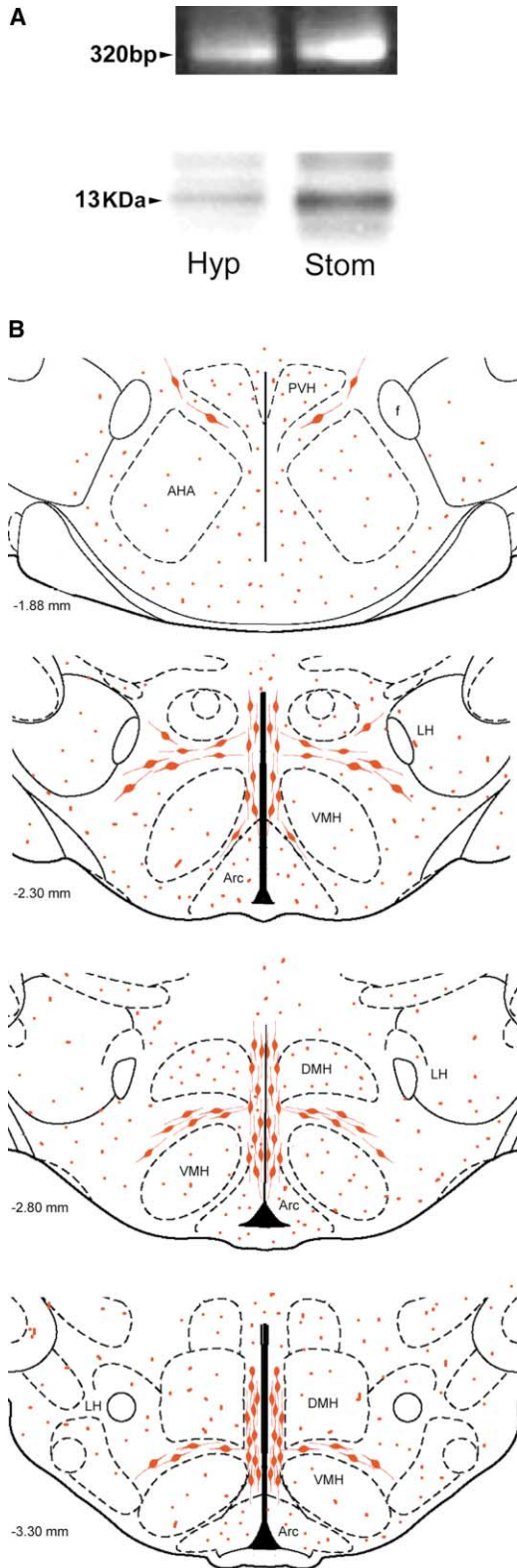LH
VMH
Arc
-2.80 mm

LH
DMH
VMH
Arc
-3.30 mm

Figure 1. Ghrelin mRNA and Peptide Expression in the Hypothalamus

(A) Ghrelin expression in the rat stomach and hypothalamus. PCR products (top) were separated on a 1.5% agarose gel stained with ethidium bromide. It should be noted that approximately four times the material was loaded onto the gel for the hypothalamus (100 ng of RT, 10 μl of PCR product loaded onto the gel) compared to the stomach (50 ng of RT, 5 μl of PCR product loaded onto the gel). Western blot analyses show (bottom) that the antibody labels a band around 13 kDa that corresponds to the size of pro-ghrelin. Note that 100 μg of hypothalamic protein (Hyp) was loaded while for the stomach 30 μg was loaded. The ghrelin level in the stomach is several fold higher than in the total hypothalamus.

(B) Schematic illustration of ghrelin-immunoreactive cell bodies (red) and projections (red dots) within the hypothalamus and adjacent areas at different distances from Bregma.

an opposite direction (Tung et al., 2001; Horvath et al., 2001; Tschop et al., 2002; Kamegai et al., 2001). The coactivation of NPY- and AGRP-dependent pathways (Kamegai et al., 2000; Shintani et al., 2001; Nakazato et al., 2001; Wren et al., 2001b; Tschop et al., 2002) by ghrelin may play an endogenous role in appetite stimulation and energy supply, since administration of ghrelin antiserum or ghrelin receptor antagonists prevents a ghrelin-induced generation of a positive energy balance (Nakazato et al., 2001). Because circulating ghrelin is predominantly derived from the stomach and intestine and may bind at hypothalamic target neurons, its putative transfer across the blood-brain barrier becomes an important factor. Surprisingly, it was observed in a mouse model that acylated ghrelin is readily transported across the blood-brain barrier in the brain-to-blood direction, but the quantity of its transport in the blood-to-brain direction appears to be negligible (Banks et al., 2002). A very recent finding revealing that vagotomy prevents peripheral ghrelin's effect on the hypothalamus (Date et al., 2002) also suggest that ghrelin's direct effect on the brain (Tschop et al., 2000; Nakazato et al., 2001) may be of intrinsic origin. We therefore investigated if, and by which mechanisms, an additional central source of ghrelin could be responsible for balancing leptin's actions in the hypothalamic regulation of energy balance.

We analyzed the expression patterns of ghrelin in the hypothalamus, the specific neuronal sites of ghrelin binding, and its electrophysiological effects on circuits previously shown to be leptin responsive. We investigated the complex actions upon neuropeptide circuits that regulate feeding and metabolism to establish an integrated concept of the role of central ghrelin in the regulation of energy homeostasis.

Results

Expression of Ghrelin in the Brain

Early studies of ghrelin mRNA distribution demonstrated expression in the brain (Kojima et al., 1999), and our RT-PCR analysis of rat stomach and hypothalamus RNA showed a 320 bp product (Figure 1A, top). This product was then sequenced and found to correspond perfectly (data not shown) with the published ghrelin sequence (Kojima et al., 1999). In further support of ghrelin production in the brain, no ghrelin mRNA was detected in the hypothalamus of ghrelin knockout mice, while their wild-type littermates showed similar expression pattern as described for the rat (data not shown).

Next, we analyzed the existence of ghrelin protein in the hypothalamus using three antisera, which had been
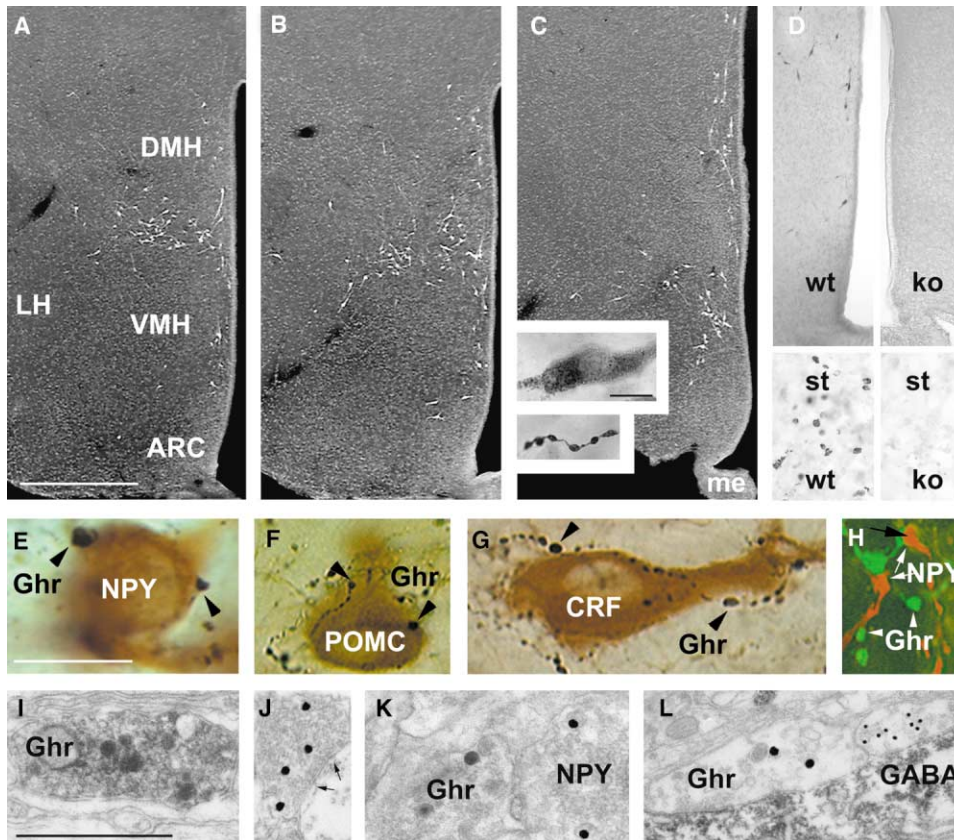
Figure 2. Ghrelin in Hypothalamic Neurons and Their Efferents

(A–C) Distribution of ghrelin-immunoreactive (IR) perikarya in an area between the arc, PVH, DMH, and VMH. Scale bar in (A) represents 300 μm (for A–D). Each image shows the immunoreactivity pattern with a different primary antiserum. Top and bottom insets in (C) are high-magnification images of a ghrelin-immunolabeled cell body and axonal varicosity, respectively. Scale bar in top inset represents 10 μm for both insets.

(D) Stomach and hypothalamus from wild-type and ghrelin knockout animals immunolabeled for ghrelin. Top left and right images show hypothalamic sections (corresponding to those depicted in A–C) from a wild-type animal and a ghrelin knockout mouse, respectively. Bottom left and right images show stomach crossections immunolabeled for ghrelin from a wild-type mouse and a ghrelin knockout littermate, respectively. Note that no specific staining could be found in either the hypothalamus (top right) or the stomach (bottom right) in ghrelin knockout animals.

(E–G) Ghrelin immunopositive axon terminals (arrowheads) in close proximity to an NPY-immunoreactive (E) and POMC-immunoreactive (F) perikarya in the arcuate nucleus and a CRF-positive cell body in the PVH (G). Scale bar in (E) represents 10 μm for (E)–(G).

(H) NPY-IR axon terminals (red fluorescence) in direct apposition to ghrelin-IR boutons (green fluorescence) in the PVH.

(I) Electron micrograph showing ghrelin immunoreactivity in dense-cored vesicles of an axon terminal. Scale bar represents 1 μm for (I)–(L).

(J) Electron micrograph showing a symmetrical synaptic membrane specialization (black arrows) between a ghrelin-immunolabeled axon terminal (25 nm immunogold in dense-cored vesicles) and an unlabeled dendrite.

(K) Electron microscopy revealed direct appositions between ghrelin (immuno peroxidase in dense-cored vesicles) and NPY-producing presynaptic terminals (25 nm immunogold in dense-cored vesicles).

(L) Direct apposition between ghrelin (25 nm immunogold particles) and GABA (10 nm immunogold particles; arrowheads) axon terminals presynaptic to an immuno-labeled TRH perikaryon in the PVH.

independently generated against ghrelin. In Western blots, these antisera labeled a band that corresponds to the nonsecreted ghrelin prohormone (13 kDa) (Figure 1A, bottom), confirming that ghrelin is produced in the hypothalamus.

Ghrelin-immunoreactive cell bodies were detected in the hypothalamus of rats and mice and were distributed in a continuum filling the internuclear space between the lateral hypothalamus (LH), arcuate (ARC), ventromedial (VMH), dorsomedial (DMH), and paraventricular hypothalamic nuclei (PVH) and the ependymal layer of the third ventricle (Figures 1B and 2A–2C; Table 1). Ghrelin knockout animals expressed no immunolabeling for

ghrelin in the hypothalamus and stomach (Figure 2D) using these antisera. In colchicine-treated animals, the intensity and number of labeled cells in this area was enhanced, which is a characteristic feature of several hypothalamic peptidergic systems. Ghrelin-immunoreactive neurons had a mostly bipolar shape and frequently exhibited rectangular appearance (Figure 2C, top inset).

Ghrelin was robustly expressed in axons (Figures 1B, 2C [bottom inset], and 2E–2L; Table 1) and was associated with dense-cored vesicles in presynaptic terminals (Figures 2I–2L). The synaptic membrane specializations were not always clear, as is the case frequently in the

Table 1. Ghrelin Immunoreactivity in the Forebrain and Diencephalon

	Cell Bodies	Processes
Thelencephalon		
Cortex	−	+
Hippocampus	−	−
Caudate-putamen	−	−
Nucleus accumbens	−	+
Central nucleus of amygdala	−	+
Cortical nucleus of amygdala	−	+
Basolateral nucleus of amygdala	−	−
Medial nucelus of amygdala	−	+
Bed nucleus of stria terminalis	−	+ +
Medial septum	−	+
Lateral septum	−	+
Diencephalon		
Organum vasculosum laminae terminalis	−	+
Medial preoptic area		
Lateral preoptic area	−	+
Anterior hypothalamus		
Periventricular area	+ +	+ + +
Supraoptic nucleus		
Suprachiasmatic nucleus	−	+
Paraventricular nucleus (magnocellular)	−	+
Paraventricular nucleus (parvicellular)	− (+)	+ + +
Lateral hypothalamus	− (+)	+ + +
Perifornical region	−	+ + +
Retrochiasmatic area	− (+)	+ + +
Arcuate nucleus	− (+)	+ + +
Dorsomedial hypothalamic nucleus	− (+)	+ + +
Ventromedial hypothalamic nucleus	− (+)	+ + +
Zona incerta	−	+
Intergeniculate leaflet of the lateral geniculate body	−	+
Thalamic paraventricular nucleus	−	+ +
Lateral habenula	−	+ +

Ghrelin cell bodies were found in the periventricular area connected with a continuum between the paraventricular, dorsomedial, arcuate, and ventromedial nuclei and lateral hypothalamus. Plus sign indicates nuclei associated with cell bodies producing ghrelin.

mediobasal hypothalamus; however, when detectable, they appeared to be Gray type II, symmetrical synaptic contacts (Figure 2J).

Ghrelin-containing axon terminals innervated several hypothalamic nuclei, including the ARC, DMH, LH, and PVH. While not the main focus of this manuscript, ghrelin projections were also detected outside the hypothalamus, including the bed nucleus of stria terminalis, medial, central, and cortical amygdale, the thalamic paraventricular nucleus, and lateral habenula (Table 1). Detailed description of ghrelin projections throughout the brain will be the focus of another manuscript.

Regarding hypothalamic systems implicated in metabolism regulation, ghrelin boutons established synaptic contacts on cell bodies and dendrites of NPY/AGRP (Figure 2E) and POMC (Figure 2F) neurons in the arcuate nucleus. Light and electron microscopic analysis with single and multiple labels also revealed that ghrelin boutons were in direct apposition to NPY and GABA axon terminals in the ARC and PVH (Figures 2H, 2K, and 2L). In the PVH, some of the ghrelin axons innervated CRH cells (Figure 2G).

Ghrelin Binding within the Brain

While data are available on the localization of GHS-R mRNA in the hypothalamus (Willesen et al., 1999; Guan et al., 1997), the subcellular localization of these recep-

tors has not been explored. To gain further insights into neuronal sites of ghrelin action, we analyzed binding of biotin-labeled ghrelin to coronal slices of the brain. Biotinylated ghrelin bound to hypothalamic regions, including the ARC (Figures 3A and 3B), LH (Figure 3C), and PVH (Figure 3E); these regions all express the GHS-R (Willesen et al., 1999; Guan et al., 1997). Ghrelin binding was also observed in the cortex (Figure 3D). Biotinylated ghrelin binding was specific, since it was prevented by pretreatment with an excess of unlabeled ghrelin (Figures 3H–3J). Ghrelin binding was predominantly localized to presynaptic boutons (Figures 3B–3E). In the hypothalamus, axon terminals that bound ghrelin frequently also contained NPY (Figures 3F and 3G). In addition, anatomical proximity of ghrelin and NPY fibers was detected throughout the hypothalamus (Figures 2H and 2K).

Electrophysiological Effects of Ghrelin

To gain further insights into the mechanism of ghrelin actions, we assessed the electrophysiological effects of ghrelin in vitro using hypothalamic slices from mice and rats, looking at NPY neurons and previously defined sites of leptin, AGRP, and NPY action in the ARC (Ahima et al., 2000; Cowley et al., 2001) and in the PVH (Powis et al., 1998). Some hypothalamic slices were prepared from transgenic mice expressing sapphire green fluo-
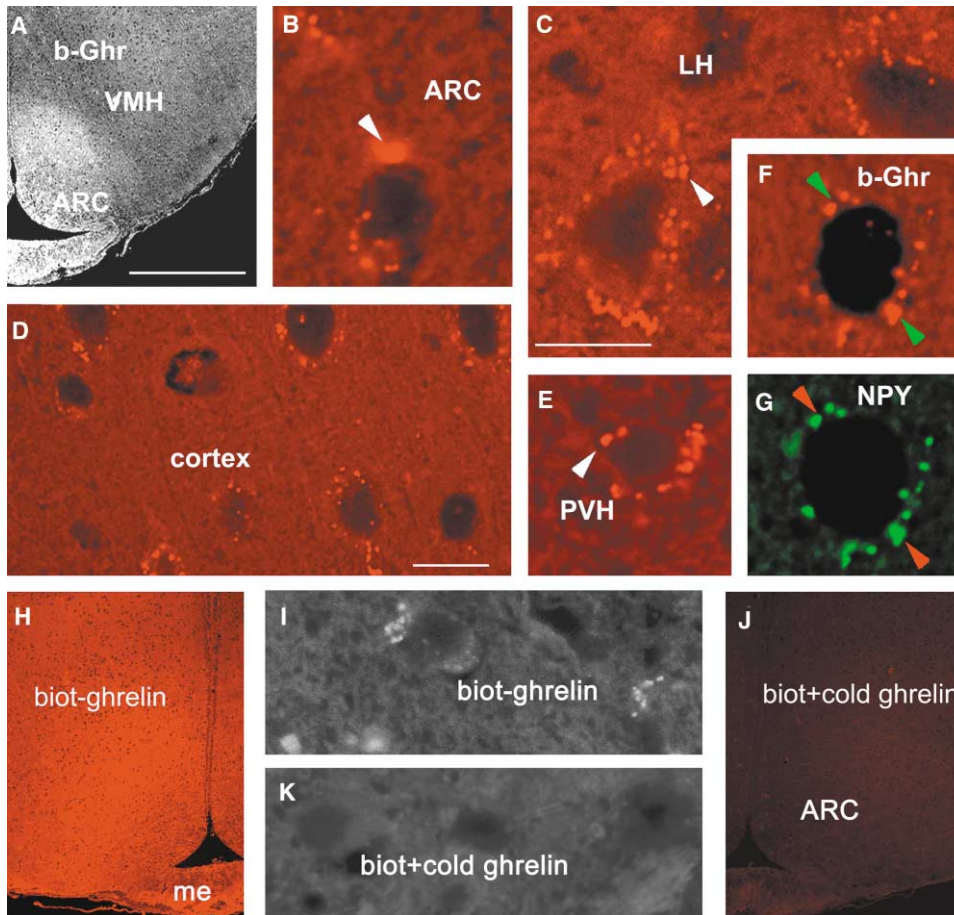
Figure 3. Ghrelin Binding throughout the Hypothalamus

(A) Biotinylated ghrelin binds to hypothalamic areas, including the ARC, DMH, and me. Scale bar represents 300 μm for (A), (H), and (J).
(B–E) Ghrelin binding is associated with axon terminals in the ARC (B), LH (C), cortex (D), and PVH (E). Arrowheads in (B), (C), and (E) point to labeled boutons. Scale bar in (C) represents 10 μm for (B), (C), and (E)–(G). Scale bar in (D) represents 10 μm for (D), (I), and (K).
(F and G) Axon terminals that bound labeled ghrelin (F, red fluorescence; green arrowheads point to some of the labeled presynaptic terminals) were frequently immunopositive for NPY (G, green fluorescence; red arrowheads point to those NPY-immunolabeled boutons that are indicated with green arrows in F).
(H–J) Biotinylated ghrelin binding (H and I) is abolished when coincubated with unlabeled ghrelin (J and K). (I) and (K) are high-magnification views of (H) and (J), respectively.

rescent protein (SFP) under the control of NPY regulatory elements (NPY-SFP mice), and these mice eutopically express SFP in NPY neurons (see Figure 4A); a detailed description of these animals is the subject of another submission.

Ghrelin (50 nM) increased the activity of NPY neurons 3.7-fold within 4 min (Figure 4B; n = 9); this increased activity was reversible with washout. This finding showed that ghrelin was likely increasing the release of NPY and AGRP at targets of arcuate NPY/AGRP neurons. Coincident with its effect on the spontaneous activity of NPY neurons, ghrelin also depolarized identified NPY neurons (11.8 $\pm$ 2.1 mV; n = 3). This dose of ghrelin was chosen because it is close to the EC_{50} for ghrelin at the GHS-R (32 nM) (Bednarek et al., 2000). Unfortunately, we were not able to identify the specific ion conductance that was modified by ghrelin, probably because of poor voltage clamp in the slice preparation.

We examined the effect of ghrelin on POMC neurons using hypothalamic slices from mice expressing green fluorescent protein under the control of proopiomelanocortin (POMC) genomic regulatory elements (POMC-GFP mice; Figure 4A; Cowley et al., 2001). In patch-clamp recordings from visually identified, arcuate POMC neurons, ghrelin (50–100 nM) treatment was observed to increase the frequency of spontaneous GABAergic inhibitory postsynaptic currents (IPSCs) onto POMC neurons (by up to 300%, mean = 149% $\pm$ 14%; n = 18; p < 0.006; Figure 4C). These IPSCs were previously shown to be due to GABA secretion from NPY neurons (Cowley et al., 2001) and correlate temporally with the increase in the activity of NPY neurons shown in Figure 4B. This increased quantal secretion of GABA onto POMC neurons was accompanied by a 65% $\pm$ 19% decrease in the spontaneous activity of POMC neurons (Figure 4D; n = 6) and a small, but significant, hyperpolarization (Figure 4E; range 0.4 to 13 mV, mean 1.47 $\pm$ 0.7 mV; p < 0.03, n = 34), consequently decreasing the frequency of action potentials in these neurons.

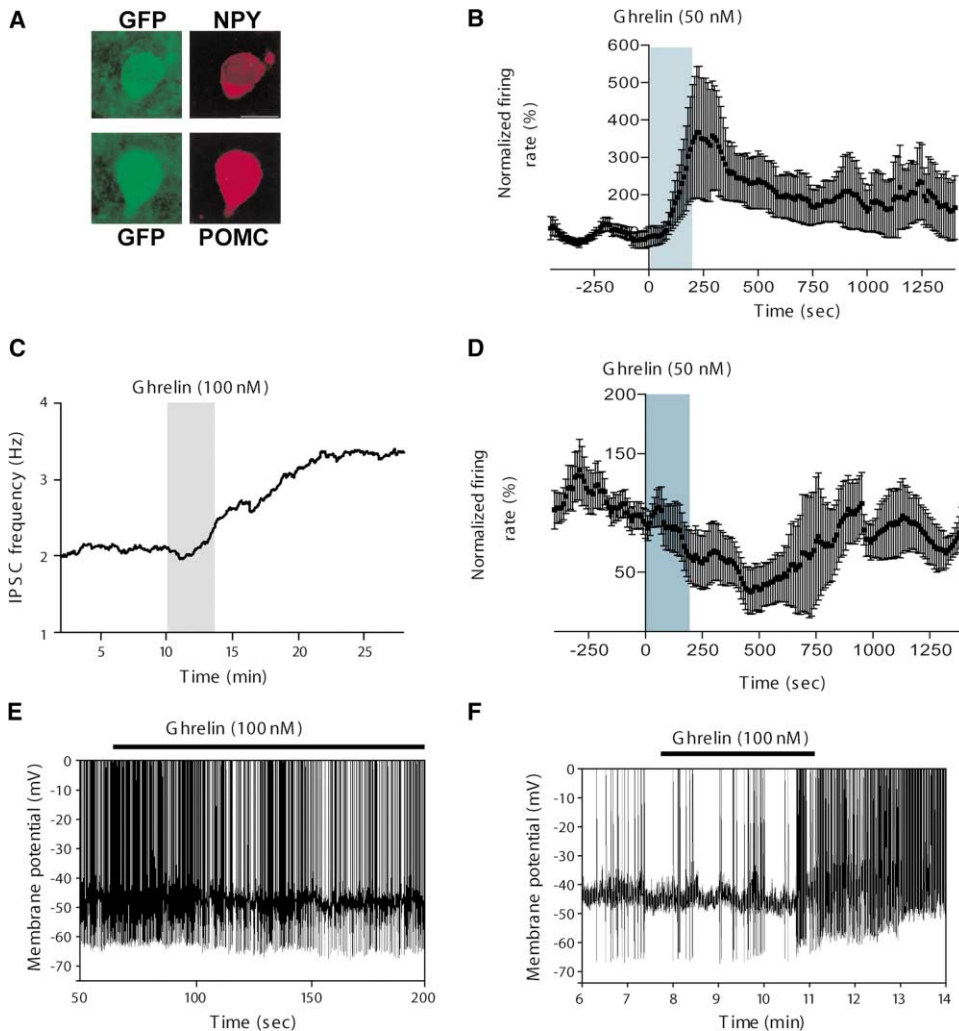Blockade of GABA$_A$ receptors (picrotoxin 100 μM) did

Figure 4. Ghrelin Increases the Spontaneous Activity of NPY Neurons and Inhibits POMC Neurons by Increasing the Inhibitory Tone onto Them

(A) Arcuate NPY and POMC neurons were selectively immunoreactive for GFP taken from two lines of transgenic mice that were analyzed in electrophysiological studies.

(B) Ghrelin (50 nM) increases the spontaneous activity of arcuate NPY neurons (n = 9).

(C) Ghrelin increases the frequency of spontaneous synaptic GABA release onto POMC neurons; figure is representative of 18 experiments.

(D) Ghrelin (50 or 100 nM) decreases the spontaneous activity of arcuate POMC neurons (n = 6).

(E) Ghrelin (100 nM) decreases the spontaneous activity of POMC neurons and causes a mild hyperpolarization; figure is representative of 34 experiments.

(F) When NPY and GABA effects upon POMC neurons are blocked (with BIBP 3026 and picrotoxin), ghrelin depolarizes POMC neurons. Figure is representative of four experiments.

not alter the ghrelin-induced hyperpolarization of the POMC neurons (1.9 ± 1.4 mV hyperpolarization, n = 9, not significant), suggesting that ghrelin was stimulating the release of another factor, most likely NPY (Cowley et al., 2001), that, in turn, would hyperpolarize the POMC neuron. Indeed, blockade of NPY Y1-receptors (the NPY receptor subtype expressed on POMC neurons) (Fuxe et al., 1997) with a specific antagonist (BIBP 3226, 500 nM) and blockade of GABA$_A$R together reversed ghrelin's hyperpolarizing effect on POMC neurons and unmasked a paradoxical, ghrelin-induced depolarization of POMC neurons (Figure 4F). This ghrelin-induced depolarization may be due to ghrelin stimulating secretion of AGRP from the NPY/AGRP/GABA terminal (Hahn et

al., 1998; Horvath et al., 1997), possibly because AGRP antagonizes the tonic inhibition of POMC neurons mediated by the auto-inhibitory melanocortin-3 receptor (MC3-R) (Cowley et al., 2001; Fong et al., 1997). In support of this premise, preliminary data suggests that AGRP (100 nM) treatment depolarizes POMC neurons (8 ± 0.8 mV, n = 3), although this data did not reach significance. In further support of the data that ghrelin activates NPY/AGRP neurons, we found that ghrelin strongly depolarized 8 of 11 non-POMC neurons (14.1 ± 3.9 mV: n = 8, p < 0.03) in the ARC of POMC-GFP mice, activating some that were previously silent.

We also analyzed the effects of ghrelin on neuronal activity of the PVH, an area targeted by efferents of ARC
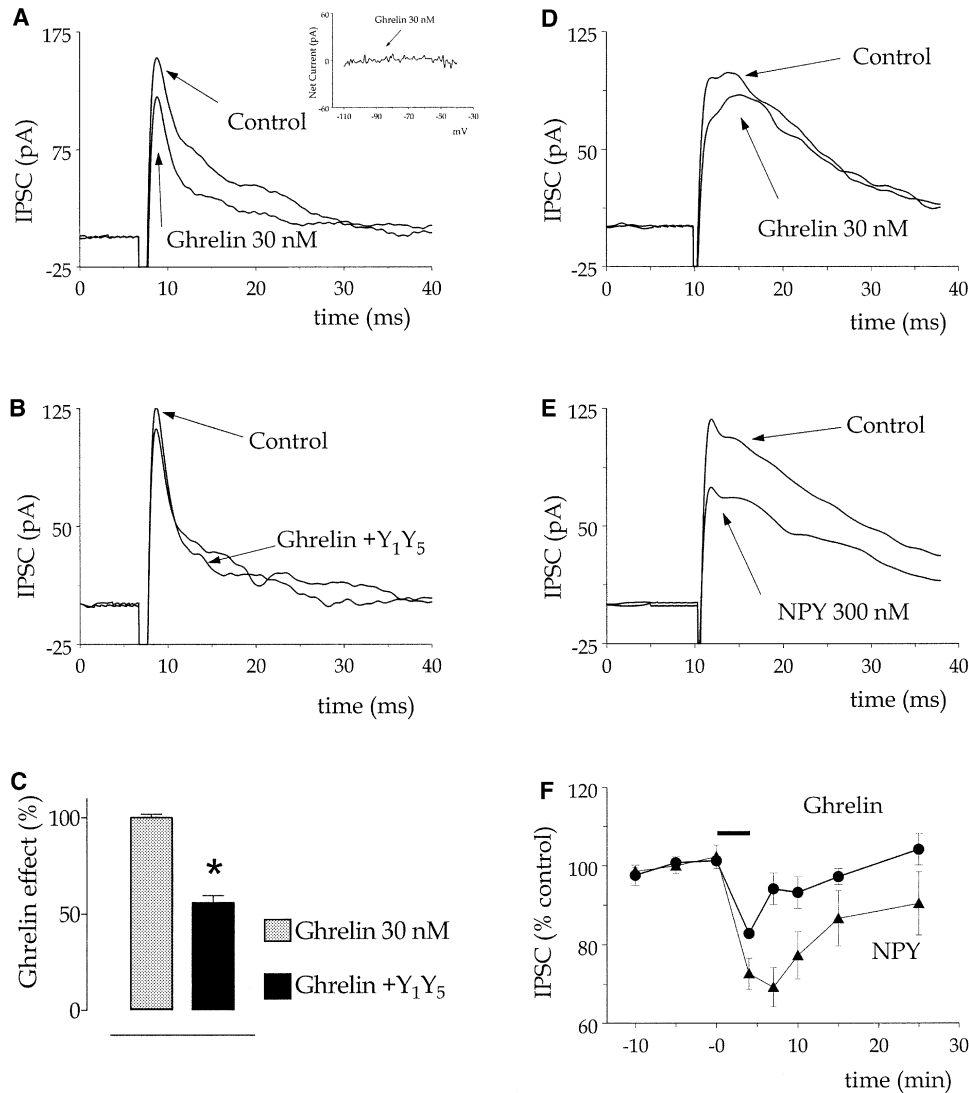
Figure 5. Electrophysiological Actions of Ghrelin in mpPVH Neurons

(A) Effect of 30 nM ghrelin on the IPSC of a mpPVH neuron. Shown superimposed are two traces, one taken in control and one in the presence of ghrelin. Inset: net (ghrelin − control) steady-state membrane current response to a voltage ramp (−110 mV to −40 mV).
(B) IPSC response to ghrelin of neuron in (A) after pretreatment with Y_1 and Y_5 receptor antagonists.
(C) The effect of ghrelin is attenuated by NPY antagonists in mpPVH neurons.
(D) Effect of ghrelin on PVH neuron IPSC.
(E) Effect of NPY on IPSC of neuron in (D).
(F) Time course of ghrelin (closed circle; 30–100 nM) and NPY (closed triangle; 100–300 nM) actions on the IPSC of PVH neurons.

NPY and POMC neurons. In conventional and perforated patch whole-cell recordings the GABAergic inputs to 73% of medial PVH neurons was inhibited (28% ± 1.5%) by ghrelin (Figure 5A), while 27% of cells showed no effect of ghrelin. We have previously shown that NPY exerts an identical effect on these neurons, which results from a presynaptic reduction of GABA release rather than a change in the response to GABA (Cowley et al., 1999). This response to ghrelin was not accompanied by a significant change in the membrane current-voltage relationship, expressed as net current (Figure 5A, inset), also consistent with a presynaptic action by ghrelin. Because ghrelin binding was colocalized at NPY-immunoreactive terminals in the PVH, we tested the hypothe-

sis that ghrelin action in the PVH results from NPY release. In the ghrelin-responsive neuron of Figure 5A, preapplication of a cocktail of NPY Y_1 and Y_5 antagonists (BIBO 3304, 500 nM; Novartis 2, 500 nM, respectively) substantially reduced or blocked the effect of ghrelin (Figure 5B). In 5 of 11 ghrelin-sensitive neurons tested, this antagonist cocktail reduced the ghrelin effect by 59% ± 12% (n = 5, p < 0.005; Figure 5C). Furthermore, in 2 of 4 ghrelin-responsive cells insensitive to the Y_1 and Y_5 antagonist cocktail, additional application of the Y_2 receptor antagonist, BIIE0246 (100 nM), reduced or blocked the ghrelin effect (not illustrated).

Application of NPY always inhibited the IPSC in ghrelin-sensitive neurons in PVH. Figure 5D shows the
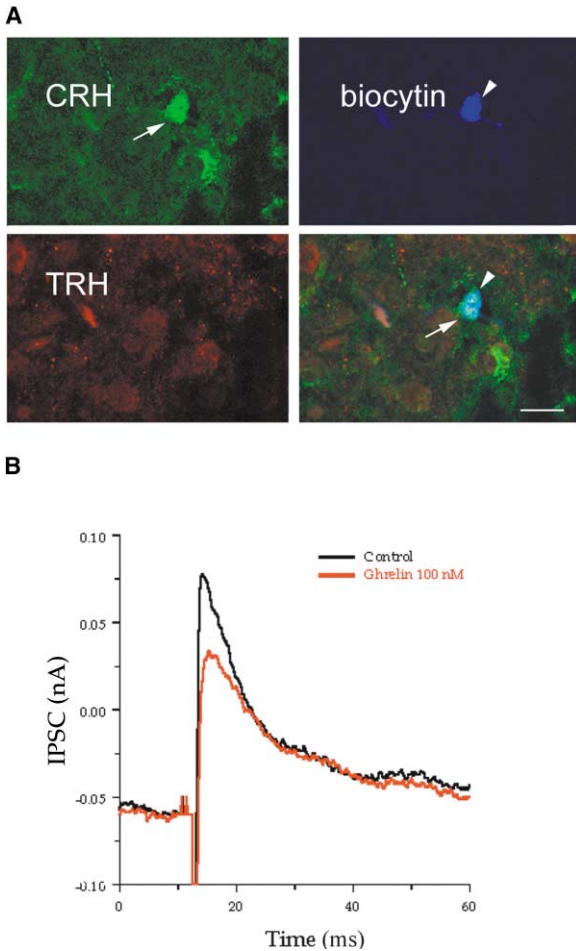
A

B

Figure 6. Triple-Label Immunocytochemistry of PVH Neurons after Perforated-Patch Recording and Electroporation

(A) A CRH-positive (green fluorescence image) but TRH immunonegative (red fluorescence image) neuron that was filled with biocytin (blue fluorescence image). Lower right is merged images of the CRH, biocytin, and TRH labelings. Scale bar represents 10 μm.
(B) Electrophysiological recordings revealed an inhibitory effect of 100 nM ghrelin on IPSC of CRH neurons.

IPSC response to ghrelin in a PVH neuron; Figure 5E shows the response to NPY in the same neuron. The magnitude and time course of the response to ghrelin (30–100 nM; n = 16) and to NPY (100–300 nM) applied to ghrelin-sensitive neurons (n = 9) is shown in Figure 5F. Thus, NPY and ghrelin act on the inputs to the same PVH neurons in the same manner.

To identify the neuropeptide phenotype of neurons that showed a ghrelin-sensitive IPSC, we filled PVH neurons (n = 20) with biocytin at the conclusion of a perforated patch electrophysiological recording and tested for colocalization of the intracellular marker with CRH and thyrotropin-releasing hormone (TRH) immunoreactivity. Eight of twenty mpPVH neurons we analyzed expressed CRH, and seven of these neurons showed a decreased IPSC in response to ghrelin (Figures 6A and 6B). Two of twenty mpPVH neurons we analyzed expressed TRH, and neither of these neurons appeared to be sensitive to ghrelin (30–100 nM) in relation to IPSC.

Discussion

Anatomy of Ghrelin Neurons
Ghrelin immunoreactive neurons were observed in a continuum filling the internuclear space between the paraventricular, arcuate, ventromedial, and dorsomedial hypothalamic nuclei, the perifornical region, and the ependymal layer of the third ventricle. The observed distribution pattern of ghrelin cells is different to previously published reports (Kojima et al., 1999) and highlights a novel cell group. Their unique distribution did not overlap with any of the known hypothalamic cell populations implicated in energy homeostasis, including those producing NPY, AGRP, POMC, melanin concentrating hormone, orexin, dopamine, and somatostatin (Chronwall et al., 1985; Haskell-Luevano et al., 1999; Oliver et al., 1977; Zamir et al., 1986; Sakurai et al., 1998; de Lecea et al., 1998; Brownstein et al., 1975). The discovery of a novel hypothalamic cell distribution argues for a unique central role for ghrelin, in addition to its role as a peripheral hormone secreted by gastric endocrine glands (Kojima et al., 1999).

The subcellular appearance of ghrelin by electron microscopy established that hypothalamic ghrelin may be secreted, and ghrelin axon terminals were found to innervate other hypothalamic peptidergic systems that are involved in metabolism regulation, including those producing AGRP and POMC in the arcuate nucleus and CRH and TRH in the paraventricular nucleus. These findings provide an anatomical basis for potential pre- and postsynaptic interactions between ghrelin and NPY/AGRP, POMC, and CRH circuits, which are critical central regulators of energy homeostasis (Kalra et al., 1999). The existence of ghrelin in axon terminals of hypothalamic regions that are important to metabolic regulation, including the ARC and PVH, suggests that ghrelin produced in the hypothalamus may modulate the activity of hypothalamic neurons to regulate energy homeostasis. The apparent anatomical proximity of ghrelin and NPY fibers further supports the concept that hypothalamic ghrelin is part of a central regulatory loop modulating orexigenic drive.

Interestingly, while ghrelin immunolabeling was mainly present in the hypothalamus, ghrelin binding was also present in extrahypothalamic sites, including the cerebral cortex, raising the possibility that either peripherally circulating ghrelin can cross the blood-brain barrier to reach these cortical sites, or that additional endogenous ligands exist for the GHS-R and/or that ghrelin may have affinity to receptors other than the currently known GHS-Rs.

Physiology of Ghrelin's Effect
The extensive apposition between terminals of hypothalamic neurons and ghrelin-expressing boutons suggested a presynaptic mode of action of ghrelin in the hypothalamus. Both the binding data and the expression of ghrelin in axons adjacent to presynaptic nerve terminals suggested that ghrelin may exert modulatory effects on neurotransmission. The dependence of ghrelin on NPY and AGRP in affecting food intake (Nakazato et al., 2001; Tschop et al., 2002; Kamegai et al., 2001; Shintani et al., 2001) and data showing colocalization
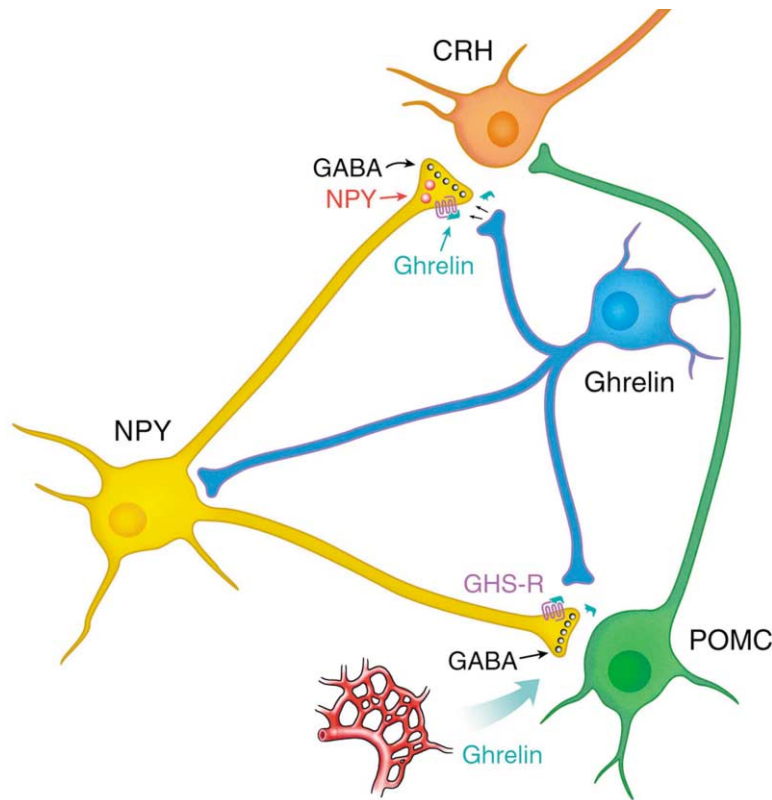
Figure 7. Schematic Drawing Summarizing the Interaction between Ghrelin and Other Hypothalamic Circuits

Model of proposed sites of ghrelin activity in the hypothalamus. The axons of hypothalamic ghrelin neurons abut NPY axons presynaptically in the arcuate nucleus and in the PVH. Ghrelin binds to, and acts via, GHS-R's to increase the activity of the terminals of NPY axons. Ghrelin increases the rate of secretion of GABA and may increase the secretion of NPY and AGRP: this altered GABA and neuropeptide secretion modulates the activity of postsynaptic POMC and CRH neurons. Ghrelin from plasma may also bind to and activate GHS-R's in the arcuate nucleus, but it is not likely to access GHS-R's in the PVH.

of NPY and c-*fos* following peripheral ghrelin administration (Wang et al., 2002) suggested that ghrelin was acting on NPY neurons and thus on many of the same circuits as does leptin (Spanswick et al., 1997). Indeed, we observed that ghrelin induced depolarization of arcuate nucleus NPY neurons. To examine the consequences of this increased NPY neuronal activity, we looked at the effects of ghrelin on POMC neurons in the ARC. Our electrophysiological observations showed that ghrelin hyperpolarizes ARC POMC neurons, an event that is most likely mediated by the GABAergic NPY/AGRP neurons. This is consistent with extensive studies of Dickson and others showing that ghrelin or growth hormone secretogogue-induced early-gene expression predominantly colocalized with NPY/AGRP neurons, rather than POMC neurons (Dickson and Luckman, 1997; Hewson and Dickson, 2000; Wang et al., 2002).

The modest inhibitory effects of ghrelin on POMC somata may not be sufficient, however, to account for the potent orexigenic effects of ghrelin (Tschop et al., 2000, 2002; Nakazato et al., 2001; Wren et al., 2001a; Kamegai et al., 2001; Lu et al., 2001). Thus, we extended our electrophysiological analysis of ghrelin to a previously characterized target site of the AGRP/NPY system in food intake regulation, the medial parvocellular PVH (Cowley et al., 1999; Pronchuk et al., 2002). This area is a target of hypothalamic ghrelin efferents (see above) but is protected by the blood-brain barrier and thus may be inaccessible to circulating ghrelin (Banks et al., 2002).

The apparent differential response of PVH CRH versus TRH cells to ghrelin is in line with the reported opposite effects of ghrelin on the hypothalamo-pituitary-adrenal and hypothalamo-pituitary-thyroid axes (Arvat et al., 2001; Wren et al., 2000). Half of the mpPVH neurons we analyzed did not have detectable colocalization with either CRH or TRH, and nine of these neurons showed a ghrelin-induced inhibition of the IPSC amplitude. It needs to be considered, however, that CRH or TRH immunolabeling in parvicellular perikarya is enhanced by colchicine pretreatment, which was not done due to its interference with electrophysiological recordings. Thus, further testing of the phenotype of the paraventricular ghrelin-sensitive neurons will be necessary to better understand ghrelin's effect in the PVH. Nevertheless, it appears that in addition to its effects on feeding, one possible effect of ghrelin is to increase the release of NPY onto GABAergic nerve terminals adjacent to CRH neurons, disinhibiting the CRH neuron and thus stimulating greater CRH release into the pituitary-hypophyseal portal circulation, driving increased ACTH secretion from the pituitary (Arvat et al., 2001).

Model of Ghrelin Action in the Brain
The results outlined here provide both anatomical substrate and physiological mechanism that can explain some of the effects of ghrelin on energy homeostasis. This work highlights a new source of ghrelin within the brain with a unique hypothalamic distribution. Interestingly, the internuclear space occupied by ghrelin-immunoreactive cells overlaps exactly the hypothalamic projections from the suprachiasmatic nucleus (Watts et al., 1987; Horvath, 1997) and the ventral lateral geniculate body of the thalamus (Horvath, 1998). Because the circadian clock resides in the suprachiasmatic nucleus and determines daily rhythms in conjunction with the ventral

lateral geniculate body, it is reasonable to suggest that the central ghrelin circuit mediates circadian/visual information to its postsynaptic targets, a proposition in line with ghrelin's meal-initiating effects (Cummings et al., 2001). The efferents of hypothalamic ghrelin neurons make synaptic contacts appropriate to enable them to alter the activity of GABAergic NPY-containing nerve terminals (Figure 7). We show that ghrelin stimulates the activity of NPY/AGRP neurons, primarily through effects at the neuron terminal. This increased activity is likely to enhance the release of neuropeptides and neurotransmitters from NPY/AGRP neurons, modulators that have been shown to act in an integrated manner to increase food intake and decrease energy expenditure. Determining how central ghrelin neurons are modulated by central and peripheral signals will be of crucial importance in understanding the role of this peptide in regulating energy homeostasis.

Experimental Procedures

All procedures using vertebrate animals (rats and mice) related to experiments of this study have been previously approved by Institutional Animal Care and Use Committees of the participating laboratories.

PCR

Four female rats (Simonsen, Gilroy, CA) were sacrificed after approximately 36 hr of fasting. Hypothalamic, cortex, and stomach RNA was isolated using TRIzol reagent according to manufacturer's instruction (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was carried out on 1 μg of total RNA for 2 hr at 37°C in a 20 μl reaction volume. PCR was performed in a 50 μl volume with 100 ng of hypothalamic and cortex RNA and 50 ng of stomach RNA (35 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min). PCR sense and antisense primers used were identical to those used by Kojima et al. (1999) (sense primer, 5′-TTG AGC CCA GAG CAC CAG AAA-3′; antisense primer, 5′-AGT TGC AGA GGA GGC AGA AGC T-3′). It should be noted that approximately four times the material was loaded onto the gel for the hypothalamus and cortex compared to the stomach. The PCR products for the stomach and hypothalamus were sequenced and found to be identical to rat ghrelin.

Immunohistochemistry

Immunolabeling for ghrelin was done using three independently generated affinity-purified polyclonal antisera against ghrelin with 0% crossreactivity with vasoactive intestinal polypeptide (INCstar, Stillwater, MA), prolactin releasing peptide (Phoenix Pharmaceuticals, Belmont, CA), galanin (Peninsula Labs, Belmont, CA), growth hormone-releasing hormone (Dr. Henryk Urbanski), neuropeptide Y (Auspep Inc, Parkville, Australia), α-MSH (Miles Scientific, Naperville, IL), β-endorphin (Chemicon, Temecula, CA), MCH (Phoenix Pharmaceuticals), and orexin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). These antisera have been shown to detect and recover synthetic ghrelin in ghrelin-spiked plasma samples (data not shown) and to recognize pro-ghrelin in Western blots of homogenates of the hypothalamus, stomach, and spleen (Figure 1B). Less intense signals in homogenates of the hypothalamus indicated lower pro-ghrelin expression levels in comparison to other ghrelin-expressing tissues.

Western blot analyses were also carried out with these antisera on intact rat hypothalamic and stomach homogenates. The antisera allowed the visualization of a band at 13 kDa, corresponding to the estimated size of pro-ghrelin. Immunolabeling with these antisera in 1:200–1:2000 dilutions using the avidin-biotin-peroxidase method and diaminobenzidine as chromogen resulted in specific labeling in hypothalamic sites that was abolished by preadsorption of the antisera with ghrelin. Furthermore, all the antisera displayed identical patterns of immunolabeling of rat hypothalamus. Data reported are from paraformaldehyde (4%) or acrolein (2%)-fixed rat tissue;

however, the same patterns of staining were seen in tissue from mice. Hypothalami of colchicine-pretreated (intracerebroventricular injection 80 μg colchicines-HCl in 20 μl saline under ketamine anesthesia 24 hr prior perfusion) animals were also analyzed for immunocytochemical labeling of ghrelin.

Double labeling immunofluorescence was carried out for ghrelin (Ghr; rhodamine-conjugated anti-rabbit IgG as secondary antisera) and NPY (mouse anti-NPY; Auspep; fluorescein-conjugated anti-mouse IgG as secondary antisera). Double immunolabeling for Ghr and NPY, Ghr and POMC (rabbit anti-β-endorphin) (Cowley et al., 2001), or Ghr and CRH (mouse anti-CRH) used the avidin-biotin peroxidase method, and a dark blue-black nickel-intensified diaminobenzidine reaction was used for the labeling of Ghr, while NPY, POMC, and CRH were labeled using the peroxidase anti-peroxidase method and a light brown diaminobenzidine reaction.

Visualization of NPY or POMC and GFP was accomplished by fluorescent labeling of either NPY or POMC using primary and secondary (rhodamine-conjugated anti-sheep or anti rabbit IgG) antisera. In some cases to further amplify GFP labeling, GFP was also visualized using mouse anti-GFP antisera that was visualized by fluorescein-conjugated anti-mouse IgG, which step then was followed by labeling for NPY or POMC as described above.

Combination of pre- and postembedding immunolabeling of ghrelin (preembedding immunoperoxidase labeling of large core vesicles in Figure 2K) and NPY (postembedding labeling of large core vesicles with 25 nm gold-conjugated secondary antisera) was used to investigate the ultrastructural relationship between Ghr and NPY (Figure 2K). In accordance with our previously published protocol (Cowley et al., 2001; Toran-Allerand et al., 2002), combination of preembedding immunoperoxidase labeling of TRH and dual postembedding labeling of Ghr (large core vesicles labeled with 25 nm gold-conjugated anti-rabbit IgG in axon terminals) and GABA (10 nm gold-conjugated anti-guinea pig IgG over small vesicles in axon terminals) was used to analyze the ultrastructural relationship between Ghr, GABA, and TRH (Figure 2L). The steps were as follows (the greatest detail is provided for this triple labeling, because it contains all the methodological steps that were used in all single and multiple labeling experiments). Incubation in rabbit anti-TRH antisera (1:1000) in PB for 48 hr at 4°C. Following several washes in PB, sections were incubated in the secondary antiserum (biotinylated goat-anti-rabbit IgG, 1:250 in PB, Vector Labs, Burlingame, CA) for 2 hr at room temperature, then rinsed in PB for 3 × 10 min and incubated for 2 hr at room temperature with avidin-biotin-peroxidase, 1:50 in PB (ABC Elite Kit, Vector Labs), followed by a diaminobenzidine (DAB) reaction (15 mg DAB, 165 μl 0.3% H₂O₂ in 30 ml PB, 5–10 min at room temperature; diffuse, electro-dense, cytoplasmic labeling) to visualize the tissue bound peroxidase. After several rinses in PB, sections were processed for postembedding labeling. Sections were dehydrated through increasing ethanol concentrations (the 70% ethanol containing uranyl acetate; for 30 min) and flat embedded in Durcupan between liquid release (Electron Microscopy Sciences, Fort Washington, PA) coated slides and coverslipped and placed in an oven to polymerize for 48 hr at 60°C. Flat embedded sections were fixed with a drop of embedding medium on the top of cylindrical Durcupan blocks and cured again for 48 hr at 60°C. Blocks were then trimmed, and ultrathin sections (60 nm on Reichert-Jung Ultramicrotome) were collected on Formvar-coated single slot grids and processed for postembedding labeling of ghrelin and GABA. These steps were carried out on Millipore-filtered solutions in humid chambers: (1) 10 min 2% periodic acid; (2) rinse in double distilled water (DDW); (3) 10 min 2% sodium metaperiodate in DDW; (3) wash in DDW; (4) 3 × 2 min rinse in pH 7.4 Tris-buffered saline containing 0.1% Triton X-100 (TBST); (5) 10 min in TBST containing 0.1% sodium borohydride and 50 mM glycine; (6) 10 min in TBST + 2% BSA; (7) incubation for 1–2 hr in rabbit anti-ghrelin diluted 1:1000 in TBST + 2% BSA; (8) 2 × 10 min wash in TBST; 10 min rinse in TBST + 2% BSA; (9) 2 hr incubation in gold-conjugated (25 nm) anti-rabbit IgG diluted 1:20 in the same buffer; (10) 3 × 3 min wash in DDW. Sections were then further immunostained for GABA. The same steps were taken as for the ghrelin immunolabeling, except that antisera against GABA was used (guinea-pig anti-GABA; 1:1000) and the secondary antisera was 10 nm gold-conjugated anti-guinea pig IgG. Sections were then contrasted with saturated uranyl acetate (10 min) and lead citrate (20–30 s). Ultrathin sections

were examined using a Philips CM-10 electron microscope. An axon terminal was considered to contain ghrelin (or GABA) if (1) the number of gold particles overlaying synaptic vesicles in a 0.5 μm^2 region of the bouton was at least three times higher than the number of gold particles in any given 0.5 μm^2 region in other compartments of the cells (these areas include regions of the cytosol in neuronal and astrocytic cell bodies and processes), and (2) the criteria of (1) could be applied to the same bouton in immediate preceding and subsequent serial sections.

Ghrelin Knockout Mice

To further assess specificity of the ghrelin antisera, we have analyzed the stomach and hypothalami of ghrelin knockout mice. Detailed description of these animals and their phenotypes is the subject of another submission. In short, mouse ghrelin genomic DNA clones were isolated from Lambda KOS genomic library using exon-4- and exon-5-specific primers. The *IRES-LacZ/MC1-Neo* selection cassette was inserted into the *ghrelin* locus as a SfiI fragment by yeast-mediated recombination to replace ghrelin exons 2 and 3 that encode ghrelin. The targeting vector consists of 3.0 kb and 4.5 kb homologous regions of genomic DNA at 5′ and 3′ of the *IRES-lacZ/MC1-Neo* cassette, respectively. The targeted ES cells and subsequently agouti pups, heterozygotes, and homozygotes were genotyped by Southern analysis using a 5′-probe, a 3′-probe, and exon probe.

Immunolabeling for ghrelin on 40 μm thick sections of the hypothalamus and stomach of knockout and wild-type animals were carried out as described above. Knockout and wild-type sections from one knockout and one wild-type animal were processed in the same vial. Three runs of such paired sections were analyzed, thus totaling three knockout and three wild-type mice.

Ghrelin Binding

Tissues were fixed overnight in zinc-buffered formalin and then transferred to 70% ethanol prior to processing through paraffin. Five micron sections were prepared and placed on positive charged slides. The slides were then baked overnight at 60°C in an oven and then deparaffinized in xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed by immersing the slides in Target Retrieval Solution (Dako Corp., Carpinteria, CA) for 20 min at 90°C (in a water bath), cooling at room temperature for 10 min, washing in water, and then proceeding with immunostaining. All subsequent staining steps were performed on the Dako Autoimmunostainer; incubations were done at room temperature, and Tris-buffered saline plus 0.05% Tween 20 (pH 7.4) (TBS, Dako Corp.) was used for all washes and diluents. Thorough washing was performed after each incubation. Slides were blocked with protein blocking solution (Dako) for 25 min; after washing, 10 $\mu g/ml$ of biotinylated ghrelin (Eli Lilly and Co., Inc., Indianapolis, IN) was then added to the slides and incubated for 60 min. A streptavidin-horseradish peroxidase kit (Dako LSAB2) was then utilized along with Alexa 568 dye (red fluorescence) to detect the bound avidin-biotin-ghrelin complexes. The slides were briefly counterstained with hematoxylin, removed from the autostainer, and dehydrated through graded alcohols to xylene. The slides were coverslipped with a permanent mounting medium and analyzed with a fluorescence microscope. Some of the biotinylated ghrelin-labled slides were further processed for fluorescent labeling of NPY using the monoclonal antisera described above and fluorescein-conjugated secondary antisera (green fluorescence). Both unlabeled ghrelin and biotin were used as negative controls, in which case no specific labeling was detected. For cold ghrelin competition with biotinylated ghrelin, we also used a modified binding assay on fresh, nonfixed hypothalamic slices. In this case, saline-perfused brains were removed, sectioned, and immediately reacted with biotinylated ghrelin (1 μM; Phoenix Pharmaceuticals) alone or in combination with equal amount of unlabeled (cold) ghrelin (1 μM; Phoenix Pharmaceuticals) for 20 min at 4°C. Subsequently, sections were fixed with 4% paraformeldehyde and reacted with avidin-Texas red and analyzed. This method is similar to that applied earlier for the analysis of binding characteristics of biotin-conjugated synthetic growth hormone secretagogues in the pituitary (Smith et al., 1997).

Electrophysiology

The effect of ghrelin on NPY neurons was determined using electrophysiological recordings from identified NPY neurons, in hypothalamic slices from NPY-Sapphire Green Fluorescent Protein mice (NPY-SFP mice). These mice express a transgene containing Sapphire GFP under the control of 150 kb of *Npy* gene regulatory elements. This transgene is the same as that used to drive the eutopic expression of CRE in NPY neurons (DeFalco et al., 2001), except that SFP has been substituted for CRE. A detailed characterization of these mice has been completed and the expression of SFP is limited to NPY neurons (unpublished). Electrophysiological recordings were made from mouse arcuate NPY neurons as previously described for POMC neurons (Cowley et al., 2001; Heisler et al., 2002; Batterham et al., 2002).

Recording were made from POMC and non-POMC neurons in 180 μm hypothalamic slices as previously described (Cowley et al., 2001; Heisler et al., 2002; Batterham et al., 2002). In preliminary experiments, we filled some neurons with neurobiotin and performed postrecording immunohistochemistry to confirm that the electrophysiological recording was made from a fluorescent neuron. In latter experiments, we followed the diffusion, and thus loss of fluorescence, of GFP or SFP from the cell into the patch electrode. Electrophysiological recordings were made from medial parvocellular PVH neurons in hypothalamic slices from rats as previously described (Cowley et al., 1999; Pronchuk et al., 2002). Slow (>4 s) voltage ramps from −110 to −40 mV were applied to all PVH neurons in control, and as appropriate, to assess possible postsynaptic consequences of all experimental manipulations. Some recordings here were made using perforated patch rather than conventional whole-cell techniques; whole-cell configuration was reached within 10 min of seal formation, with 9–10 MOhm access resistance. The electrode solution for perforated patch recordings contained (in mM) K Gluconate, 125; MgCl$_2$, 5; HEPES, 10; EGTA, 10; Nystatin, 1.5%; biocytin, 1.5%). At the end of perforated patch recording experiments, biocytin in the pipette solution was electroporated through the patch by depolarizing voltage clamp pulses (60 mV, 100 ms, 5 Hz) applied for 5–6 min while the cell was held at −60 mV, to post hoc identify the PVH neurons. The slice was then fixed overnight in a 4% paraformaldehyde-sodium phosphate-buffered saline (PBS, pH 7.4) fixative at 4°C, then washed three times for 10 min in 0.02 M potassium PBS (KPBS) and dehydrated at 4°C in 20% sucrose overnight. Thin (20 μm) sections were cut with a cryostat (Leica, Jung Frigocut 2800E), collected into 0.02 M KPBS, and washed three times for 10 min in KPBS. Sections were then incubated for 72 hr at 4°C in a mixture of anti-CRF (1:3000, guinea pig, Peninsula Laboratories) and anti-TRH (1:3000, rabbit, Dr. Eduardo Nillni, Brown University), diluted in 0.02 M KPBS with 0.3% Triton X-100 (Sigma) and 2% normal goat serum (Rockland). Sections were then washed five times for 10 min and then incubated for 50–60 min in a mixture of secondary antibodies comprising anti-rabbit ALEXA 568 (1:200), anti-guinea pig ALEXA 488 (1:200), and streptavidin-conjugated ALEXA 350 (1:200, to reveal biocytin labeling) (all from Molecular Probes, Eugene, OR), diluted in 0.02 M KPBS with 0.3% Triton X-100 and 2% normal goat serum. Sections were mounted in Prolong anti-fading medium (Molecular Probes), and images were taken and processed using a Zeiss LSM510 confocal microscope. Data were analyzed by ANOVA or Wilcoxon's Signed Rank Test (for nonparametric data) using Prism (GraphPad Software, San Diego, CA).

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