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Ghrelin-Induced Food Intake Is Mediated via the Orexin Pathway

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The hypothalamus regulates energy intake by integrating the degree of starvation or satiation with the status of the environment through a variety of neuronal and blood-derived signals. Ghrelin, a peptide produced in the stomach and hypothalamus, stimulates feeding and GH secretion. Centrally administered ghrelin exerts an orexigenic activity through the neuropeptide Y (NPY) and agouti-related protein systems. The interaction between ghrelin and other hypothalamic orexigenic peptides, however, has not been clarified. Here, we investigated the anatomical interactions and functional relationship between ghrelin and two orexigenic peptides, orexin and melanin-concentrating hormone (MCH), present in the lateral hypothalamus. Ghrelin-immunoreactive axonal terminals made direct synaptic contacts with orexin-producing neurons. Intracerebroventricular administration of ghrelin induced Fos expression, a marker of neuronal activation, in orexin-producing neurons but not in MCH-producing neurons. Ghrelin remained competent to induce Fos expression in orexin-producing neurons following pretreatment with anti-NPY IgG. Pretreatment with anti-orexin-A IgG and anti-orexin-B IgG, but not anti-MCH IgG, attenuated ghrelin-induced feeding. Administration of NPY receptor antagonist further attenuated ghrelin-induced feeding in rats treated with anti-orexin-IgGs. Ghrelin-induced feeding was also suppressed in orexin knockout mice. This study identifies a novel hypothalamic pathway that links ghrelin and orexin in the regulation of feeding behavior and energy homeostasis. (*Endocrinology* 144: 1506–1512, 2003)

GHRELIN WAS ORIGINALLY isolated from human and rat stomach as a cognate endogenous ligand for the GH secretagogue receptor (GHS-R; Ref. 1). This 28-amino-acid peptide has a posttranslational *n*-octanoyl modification indispensable for its activity. Ghrelin stimulates GH release when peripherally or centrally administered to rats and when applied directly to rat primary pituitary cells (1–3). In addition, ghrelin administration increases food intake and body weight gain (3–9). Whereas ghrelin secretion is up-regulated under negative energy balance conditions, including starvation, insulin-induced hypoglycemia, cachexia, and anorexia nervosa, it is down-regulated under conditions of positive energy balance, such as feeding, hyperglycemia, and obesity (10–14). Gastric ghrelin enters the brain across the blood-brain barrier (15). Recently, stomach-derived ghrelin's signals for starvation has been reported to be relayed to the hindbrain via the vagus afferent nerve (16).

Although ghrelin is predominantly produced in endocrine cells of the stomach (17, 18), it is also synthesized in the hypothalamic arcuate nucleus (1, 19), a critical region for feeding. The ghrelin receptor, however, is extensively distributed throughout the brain to areas such as the lateral hypothalamus (LH) and arcuate nucleus (20, 21). Both areas contain several subsets of neurons that produce neuropeptides implicated in feeding regulation, including neuropeptide Y (NPY), agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript, proopiomelanocortin, melanin-concentrating hormone (MCH), and orexins (orexin-A and orexin-B), which are also termed hypocretins (22, 23). Centrally administered ghrelin may interact with these peptides to regulate food intake and energy homeostasis. Although the mechanism of ghrelin's orexigenic activity is related to the NPY and AgRP pathways (6, 7, 24), the interaction of ghrelin with other energy-regulating systems is unclear.

Orexin-A and -B, produced from the 130-amino-acid prepro-orexin precursor in the LH, have a 46% amino acid sequence identity and stimulate food intake (25). MCH, which is a 19-amino-acid neuropeptide and whose neurons are coextensive but not colocalized with orexin neurons in the LH (26), also stimulates feeding when centrally administered (27). Using electron microscope immunohistochemistry and immunofluorescence microscopy, we investigated the anatomical distributions of ghrelin with orexin and MCH. We also examined the ghrelin-induced expression of c-*fos*, a marker of neuronal activation (28), in orexin- and MCH-expressing neurons. To investigate the functional relationship between ghrelin and orexins or MCH, we examined the

Abbreviations: ABC, Avidin-biotin complex; AgRP, Agouti-related protein; DAB, 3,3′-diaminobenzidine tetrahydrochloride; GHS-R, GH secretagogue receptor; icv, intracerebroventricular; LH, lateral hypothalamus; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; SGI, silver-gold-intensification.

effect of pretreatment with anti-orexin-A and -B IgGs or anti-MCH IgG on ghrelin-inducing feeding. We also studied ghrelin-induced food intake in orexin knockout mice. We demonstrate that ghrelin interacts with the orexin system to induce feeding.

Materials and Methods

Animals

Animals were housed individually in plastic cages at 22 ± 1 C in a 12-h light, 12-h dark cycle (light on at 0700–1900 h) and were given standard laboratory chow and water *ad libitum*. Male Wistar rats weighing 300–350 g (Charles River Japan, Inc., Shiga, Japan), orexin knockout mice (12-wk-old male) that were generated by targeted mutation in embryonic stem cells (29), and wild-type littermates were used in accordance with the guidelines of the Japanese Physiological Society for animal care. Following anesthesia by ip injection of sodium pentobarbital (Abbot Laboratories, Chicago, IL), an intracerebroventricular (icv) cannula was implanted into the lateral cerebral ventricle as described (30, 31). Proper placement of the cannulae was verified upon completion of the experiment by dye administration. Only animals demonstrating progressive weight gain after surgery were used in subsequent experiments.

Electron microscope immunohistochemistry

Forty-eight hours before perfusion, three Wistar rats were injected with colchicine (200 μg per rat) in the lateral ventricles to increase the immunostaining of ghrelin- or orexin-expressing neurons. Following anesthesia with an ip injection of sodium pentobarbital, rats were perfused through the ascending aorta for 10 min with 100 ml 0.9% saline, then for 40 min with 500 ml fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brain was removed immediately and postfixed in fixative for 2–4 h at 4 C. The brain was cut into 30- to 40-μm thick sections using an Oxford vibratome (Oxford Instruments, Abingdon, UK). Sections were incubated for 12 h with rabbit anti-ghrelin antiserum (no. G606, final dilution 1:32,000; Ref. 17) at 4 C and visualized by the avidin-biotin complex (ABC) method (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA) using 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St Louis, MO) and 0.005% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6). Sections were subjected to either direct observation under a light microscope or silver-gold-intensification (SGI; Ref. 19). Sections treated for SGI were incubated with goat anti-orexin-A antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; dilution 1:20,000) for 24 h at 4 C, and then visualized by ABC. Orexin-A labeling was performed using DAB without SGI. For examination by electron microscopy, the sections were postfixed with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4 C, dehydrated

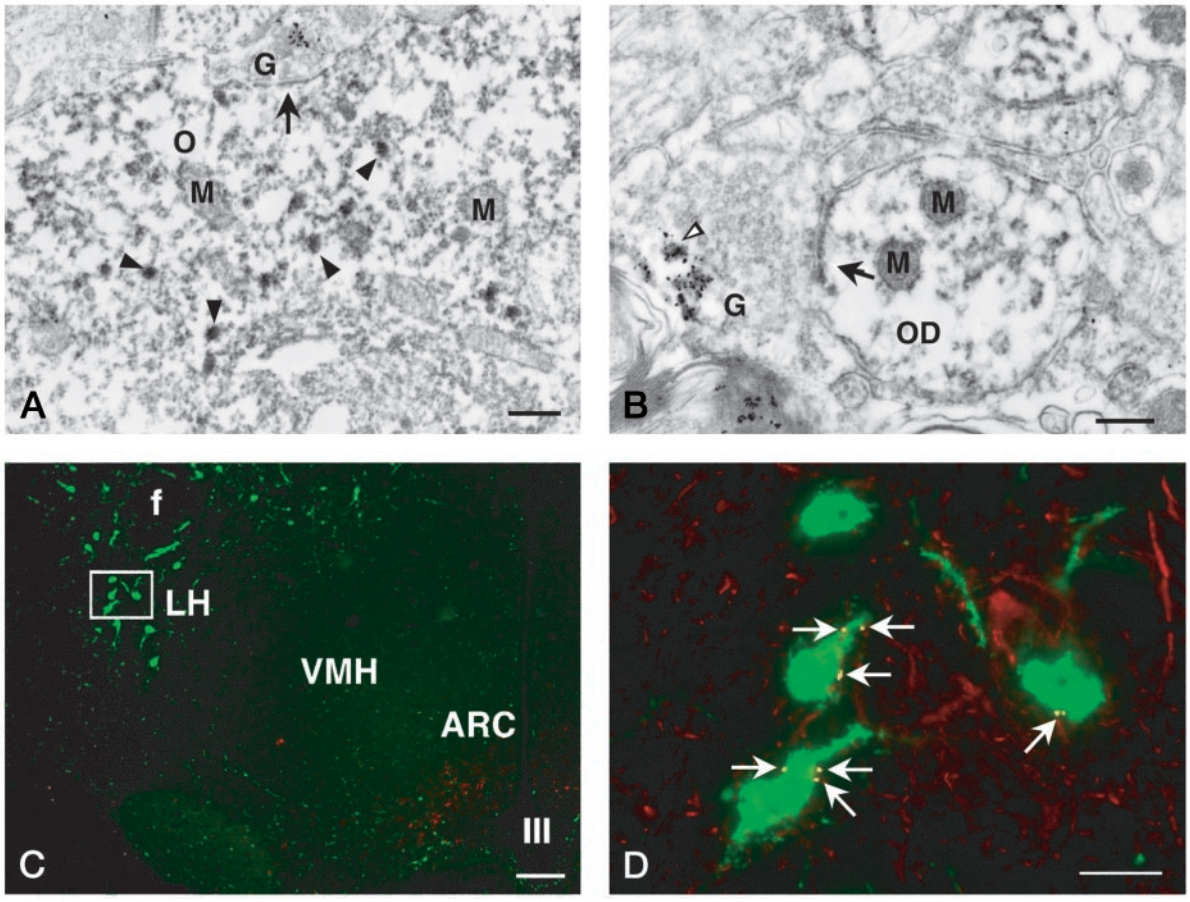

FIG. 1. The innervation of ghrelin-immunoreactive axons to orexin-producing neurons. A and B are electron micrographs, whereas C and D are immunofluorescent micrographs. A, A ghrelin-immunoreactive axon terminal (G) makes a synapse (*arrow*) on an orexin-immunoreactive perikaryon (O). The perikaryon contains many dense-cored vesicles (*closed arrowheads*) immunopositive for orexin. M, Mitochondria. B, A ghrelin-immunoreactive axon terminal (G) makes a symmetrical synapse (*arrow*) on an orexin-immunoreactive dendrite (OD). Ghrelin-immunoreactive axon terminal contains immunopositive dense-cored vesicles (*open arrowhead*). C, Ghrelin-producing neurons (*red* fluorescence) and orexin-producing neurons (*green* fluorescence) are localized to the arcuate nucleus and LH, respectively. D, A higher magnification of an area outlined in C. Ghrelin-immunoreactive fibers (*red*) are found in close proximity to orexin-producing neurons (*green*). *Arrows* indicate the apposition of the ghrelin fibers to orexin neurons. ARC, Arcuate nucleus; f, fornix; VMH, ventromedial hypothalamus; III, third ventricle. *Scale bars*, A, 0.2 μm; B, 0.2 μm; C, 100 μm; D, 10 μm.

in a graded series of ethanol concentrations, and embedded in Epon-Araldite (Structure Probe, Inc., West Chester, PA). Ultrathin sections were cut and examined under a H-7000 electron microscope (Hitachi, Tokyo, Japan). As a control, anti-ghrelin antiserum was either omitted or replaced by normal rabbit or goat serum.

Immunofluorescence double staining

Following perfusion with 2% paraformaldehyde, the brains of three Wistar rats were removed and immersed for 12 h in fixative at 4 C. Samples were then transferred into a 30% solution of sucrose. Brains were quickly frozen in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and cut into 7-μm-thick coronal sections on a cryostat (Microm HM 500; Microm, Heidelberg, Germany). Sections were incubated for 2 d with goat anti-orexin-A antiserum (dilution 1:10,000) at 4 C, then with Alexa 488-conjugated donkey antigoat IgG antibody (Molecular Probes, Inc., Eugene, OR; dilution 1:400) for 2 h. After washing with phosphate buffer saline (pH 7.4), samples were incubated for 2 d with a rabbit anti-ghrelin antibody at 4 C. Slides were then incubated with Alexa 546-labeled goat antirabbit IgG antibody (Molecular Probes, Inc; dilution 1:400). Samples were observed under an Olympus AX-70 fluorescence microscope (Olympus Co. Ltd., Tokyo, Japan).

Fos expression

Ghrelin (Peptide Institute, Inc., Osaka, Japan; 500 pmol/10 μl saline) or saline was injected icv to rats (n = 3 per group) 90 min before transcardial perfusion with 4% paraformaldehyde fixative. Also, rats (n = 3 per group) were administered anti-NPY IgG (Peptide Institute Inc.; 0.5 μg/5 μl saline) or control serum IgG (0.5 μg/5 μl saline) icv 3 h before icv ghrelin (500 pmol/10 μl saline) administration. The amount of anti-NPY IgG, 0.5 μg, was sufficient to suppress ghrelin-induced feeding (7). Following transcardial perfusion 90 min later, the tuberal hypothalamus was cut into 40-μm-thick sections. Sections were incubated for 2 d with goat anti-Fos antiserum (Santa Cruz Biotechnology, Inc.; dilution 1:1500), then stained by ABC. A proportion of the hypothalamic sections were additionally stained with either rabbit anti-orexin-A antiserum (32) or rabbit anti-MCH antiserum (Phoenix Pharmaceuticals, Inc., Belmont, CA; dilution 1:200). We quantitated the number of neurons with orexin or MCH and Fos colocalization under a light microscope by counting two randomly selected visual fields in two sections from each rat.

Food intake

Rabbit anti-orexin-A and anti-orexin-B antisera were produced as described elsewhere (32). We purified anti-orexin-A IgG and anti-orexin-B IgG by Affi-gel protein A affinity (Bio-Rad Laboratories, Inc., Hercules, CA) and either CNBr-Sepharose-coupled (Bio-Rad Laboratories, Inc.) orexin-A or orexin-B affinity chromatography. We determined the quantity of purified IgG using a DC protein assay kit (Bio-Rad Laboratories, Inc.). Because anti-orexin-A IgG did not cross-react with orexin-B and anti-orexin-B did not cross-react with orexin-A, these two IgGs were given concurrently for an icv administration. Rats (n = 8–10 per group) were given an icv administration of anti-orexin-A IgG (0.25 μg) and anti-orexin-B IgG (0.25 μg), anti-MCH IgG (0.5 μg), or control serum IgG (0.5 μg) at 1900 h. We then measured dark-phase food

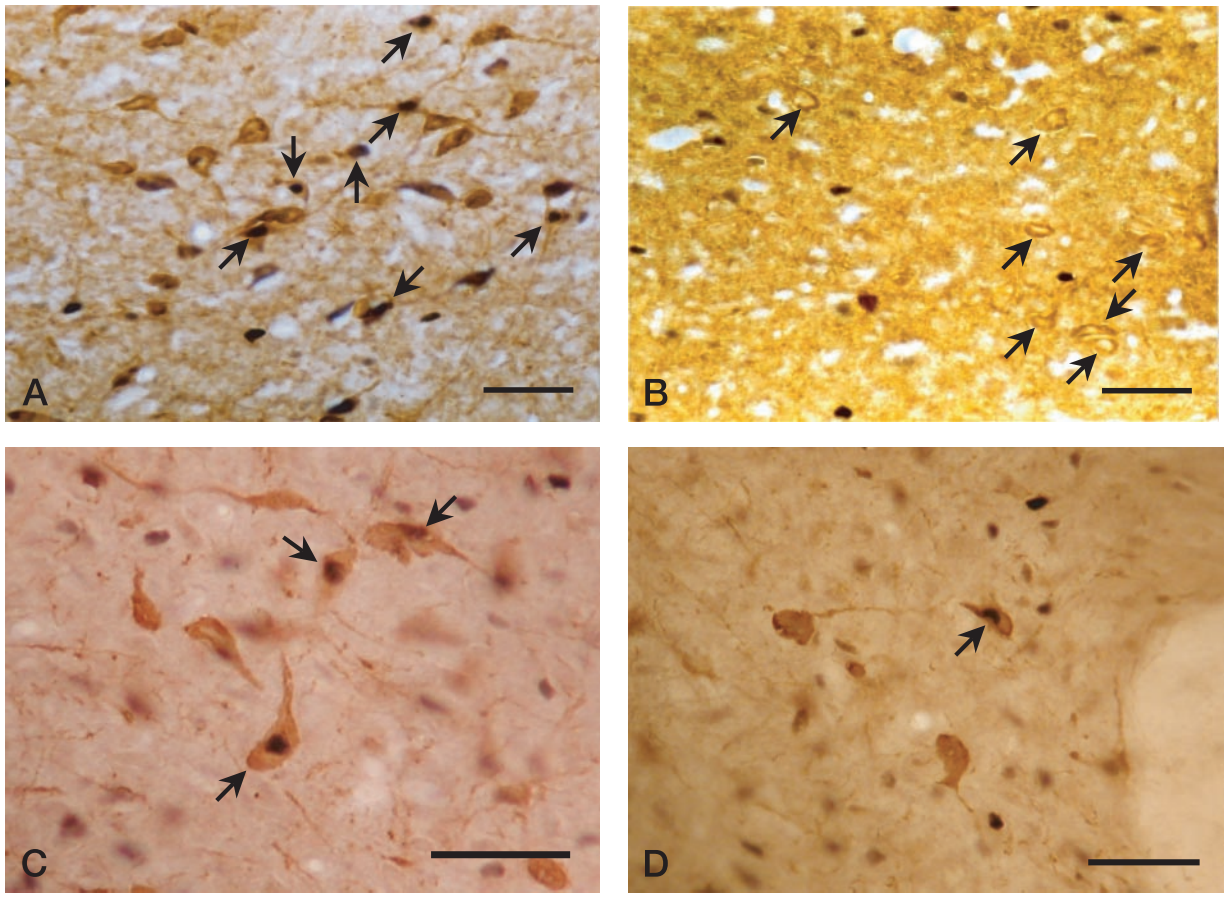

FIG. 2. Fos expression determined by immunohistochemistry in the LH following icv administration of ghrelin. A, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in the neurons of rats given ghrelin. B, Fos (*dark blue-black*) is not expressed in MCH-containing neurons (*arrows; brown*) following ghrelin administration. C, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in ghrelin-treated rats following anti-NPY IgG (0.5 μg) administration. D, Costaining (*arrows*) of Fos (*dark blue-black*) with orexin (*brown*) in ghrelin-treated rats following control IgG (0.5 μg) administration. Rats received 500 pmol ghrelin. *Scale bars*, 50 μm.

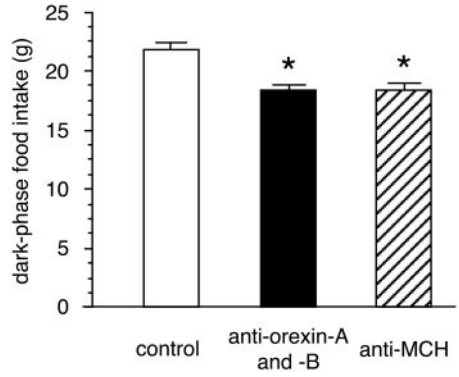

FIG. 3. The effect of icv administration of anti-orexin-A (0.25 μg) and -B (0.25 μg) IgGs or anti-MCH IgG (0.5 μg) on 12-h dark phase food intake from 1900 to 0700 h. *, $P < 0.001$ (*vs.* control IgG).

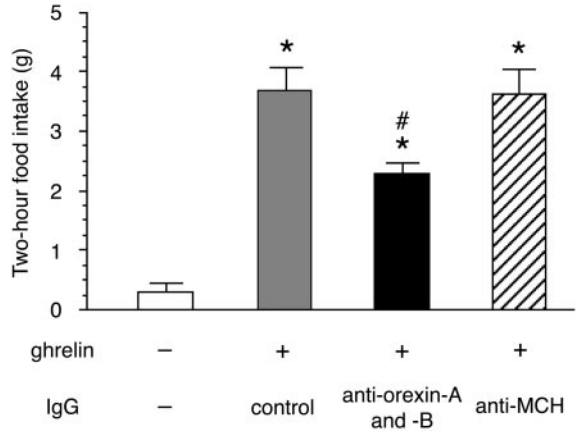

FIG. 4. The effect of the administration of anti-orexin-A (0.25 μg) and -B (0.25 μg) IgGs or anti-MCH IgG (0.5 μg) on ghrelin-induced feeding. A, Two-hour food intake was measured in ghrelin-treated (200 pmol) rats following either anti-orexin-A and -B IgGs or control IgG. B, Two-hour food intake in ghrelin-treated (200 pmol) rats following anti-MCH IgG or control IgG. *, $P < 0.001$ (*vs.* nontreatment). #, $P < 0.05$ (*vs.* ghrelin + control IgG).

intake for 12 h. Ghrelin (200 pmol) was injected icv at 1100 h to rats (n = 10–12 per group) 3 h after icv administration of anti-orexin-A and -B IgGs (0.25 μg each), anti-MCH IgG (0.5 μg), or control serum IgG (0.5 μg), after which 2-h food intake was measured. Ghrelin (200 pmol) was also administrated to rats (n = 6–8 per group) icv concurrently with either an NPY receptor antagonist, 1229U91 (30 μg, Y1 NPY receptor antagonist) (33), the kind gift of Banyu Pharmaceuticals (Tokyo, Japan), or control serum IgG, 3 h after icv administration of either anti-orexin-A and -B IgGs (0.25 μg each) or control serum IgG (0.5 μg). Ghrelin (200 pmol/2 μl saline) was also administered icv to orexin knockout mice (n = 7) and wild-type littermates (n = 7). Following injection, rats and mice were returned to their cages and food intake was measured for 2 h after treatment.

Statistical analysis

The means ± SEM were determined by one-way ANOVA, followed by the unpaired *t* test. Differences were considered to be significant when $P < 0.05$.

Results

Immunoelectron microscopy and immunofluorescence double staining

Ghrelin-immunoreactive neuronal axons and terminals contained large dense-cored synaptic vesicles, indicated by DAB-SGI reaction products (Fig. 1, A and B). Orexin expression was visualized by DAB-labeled structures, seen as a *light-to-dark gray* (Fig. 1, A and B). Orexin-producing neurons and their dendritic processes often received synapses from ghrelin-containing axon terminals (Fig. 1, A and B). No positive DAB reaction products were detected when anti-ghrelin antiserum was omitted or replaced by normal rabbit or goat serum (data not shown).

Ghrelin-expressing neurons were predominantly found in the hypothalamic arcuate nucleus, whereas orexin-producing neurons were restricted to the LH (Fig. 1C). Dense populations of ghrelin-containing fibers and a small number of orexin-containing fibers were identified in the arcuate nucleus (Fig. 1C). Ghrelin fibers were also found in the LH (Fig. 1D). Subpopulations of ghrelin-immunoreactive terminals directly contacted orexin-containing neurons in the LH (Fig. 1D).

Fos expression

Following icv administration of ghrelin, Fos-immunoreactive neurons were observed in the LH. Ghrelin was found to induce Fos expression in 23 ± 8% of orexin-immunoreactive neurons by double immunohistochemistry (Fig. 2A). Ghrelin did not, however, induce Fos expression in MCH-immunoreactive neurons (Fig. 2B). Despite pretreatment with anti-NPY or control serum IgG, ghrelin remained capable of inducing Fos expression in orexin-immunoreactive neurons (Fig. 2, C and D).

Effects of orexins and MCH on ghrelin-induced feeding

We coadministered anti-orexin-A IgG and anti-orexin-B IgG to block the activities of orexins. We first examined the effect of anti-orexin-A and -B IgGs or anti-MCH IgG on the suppression of feeding. They significantly reduced dark-phase food intake in comparison with control IgG treatment (Fig. 3). We next investigated the effect of endogenous orexin or MCH blockade against ghrelin-induced feeding. Ghrelin increased 2-h food intake in rats following icv administration of control IgG (Fig. 4). Pretreatment with anti-orexin-A and -B IgGs, however, reduced ghrelin-induced food intake to

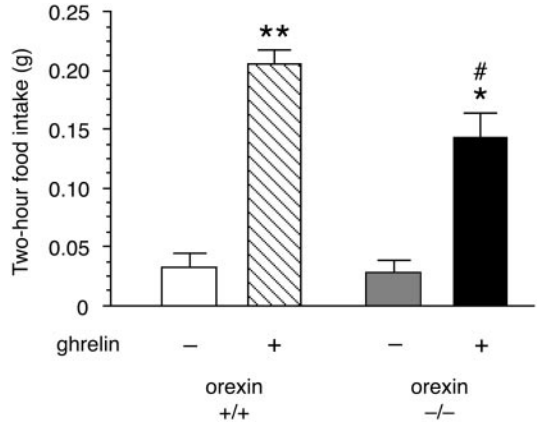

FIG. 5. The effect of icv ghrelin (200 pmol) or vehicle injection on feeding in orexin knockout mice (−/−) and wild-type littermates (+/+). *, $P < 0.01$; **, $P < 0.001$ (*vs.* control vehicle). #, $P < 0.05$ (*vs.* wild-type littermates).

one half of the level seen in rats given control IgG + ghrelin (Fig. 4). Pretreatment with anti-MCH IgG did not affect ghrelin-induced feeding (Fig. 4). Ghrelin-induced food intake in orexin-deficient mice was significantly reduced in comparison with wild-type littermates (Fig. 5). Y1 NPY receptor antagonist did not affect 2-h food intake. Coadministration of ghrelin and Y1 NPY receptor antagonist also reduced ghrelin-induced feeding by 41% (Fig. 6). The combination of ghrelin and Y1 NPY receptor antagonist coadministration with an icv administration of anti-orexin-A and -B IgGs additively reduced ghrelin-induced feeding by 87% (Fig. 6).

Discussion

This study demonstrates the synaptic contact of ghrelin-containing axons with orexin-producing neurons in the rat hypothalamus. In addition, we identified a functional interaction between ghrelin and orexins. Icv administration of ghrelin stimulates both GH secretion and food intake (1–9); ghrelin-induced feeding is thought to occur independently of GH, as icv administered ghrelin also increased food intake in spontaneous GH-deficient rats (6, 7). Ghrelin stimulates feeding through NPY and AgRP (6, 7), orexigenic peptides colocalized in neurons of the hypothalamic arcuate nucleus (34). Ghrelin receptor mRNA is expressed in NPY/AgRP neurons of the arcuate nucleus (35). Centrally administered ghrelin induced Fos expression in approximately 40% of NPY/AgRP neurons (7) and increased NPY and AgRP mRNA levels (5–7, 24).

Ghrelin receptor mRNA is also present in the LH of rats (21), a region implicated in the regulation of feeding behavior and energy homeostasis. Animals with LH lesions exhibit decreased food intake (36, 37), whereas electrical stimulation of the LH during a satiated state promotes feeding (37, 38). Orexin and MCH are orexigenic neuropeptides synthesized specifically in the LH (22, 25–27, 32, 39, 40). Ghrelin fibers project to the LH to synapse on orexin-immunoreactive neurons. Icv administration of ghrelin induced Fos expression in orexin-expressing neurons but not in MCH-expressing neurons; this result is consistent with a recent finding that icv administration of GHRP-6, a synthetic GH-releasing hexapeptide that binds to the ghrelin receptor (41), induced Fos in a similar neuronal pattern (9). These findings indicate that ghrelin may stimulate feeding through both the orexin system and the NPY and AgRP systems.

Several peptidergic and monoaminergic systems that participate in the regulation of feeding and energy homeostasis in the hypothalamus link through neuronal circuits. NPY fibers directly project to orexin neurons (42, 43); icv injection of anti-orexin antiserum before NPY injection significantly attenuated NPY-induced feeding (44), indicating that NPY interacts with orexin both anatomically and functionally. As ghrelin induces feeding through activation of the NPY system, we sought to investigate the activation of orexin-producing neurons by ghrelin via the NPY system. We therefore examined ghrelin-induced Fos expression in orexin-producing neurons following pretreatment with anti-NPY IgG. Ghrelin remained competent to induce Fos expression in orexin-producing neurons following pretreatment with anti-NPY IgG, suggesting that ghrelin activates orexin-producing neurons in a manner independent of NPY.

Orexin regulates not only feeding and energy homeostasis but also sleep-wakefulness, neuroendocrine homeostasis, and autonomic functions (25, 29, 40, 45, 46). Ghrelin induced Fos expression in approximately 23% of orexin-immunoreactive neurons. As 33% of orexin-immunoreactive neurons are glucosensitive (47) and a subset of these neurons express the receptor for leptin (42), a satiation signal produced in the adipocytes, a part of orexin-expressing neurons may play a crucial role in the regulation of feeding and energy homeostasis.

To investigate the functional relationship of ghrelin with either the orexins or MCH, we examined the effect of anti-orexin-A and -B IgGs or anti-MCH IgG pretreatment on ghrelin-induced feeding. Food intake induced by ghrelin was attenuated by anti-orexin-A and -B IgGs, but not anti-MCH IgG pretreatment. In addition, ghrelin-induced food intake in mice deficient in orexin was significantly lower than that seen in wild-type littermates. These data suggest that centrally administered ghrelin increases food intake through the action of the orexin system. To date, six functional NPY receptors (Y1–Y6) have been identified (48, 49). NPY stim-

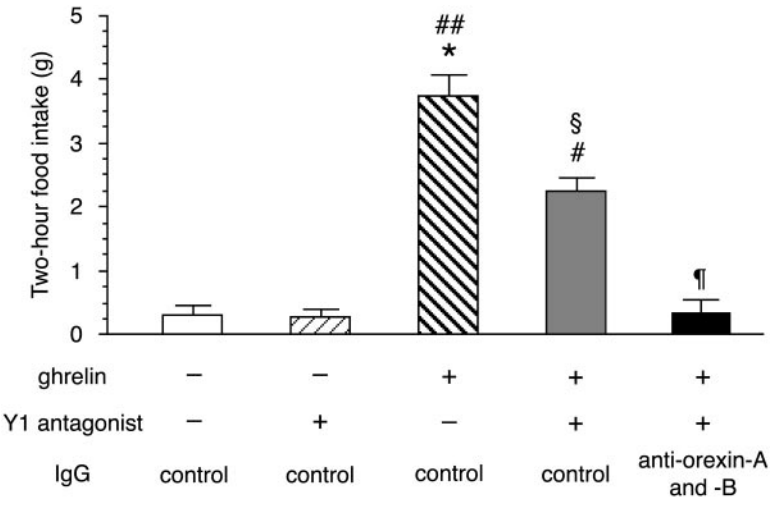

FIG. 6. Suppressive effect of Y1 antagonist (Y1 NPY receptor antagonist) and anti-orexin-A (0.25 μg) and -B (0.25 μg) IgGs on ghrelin-induced feeding. *, $P < 0.001$ (*vs.* control vehicle + control IgG). #, $P < 0.01$; ##, $P < 0.001$ (*vs.* Y1 antagonist + control IgG). §, $P < 0.001$ (ghrelin + control IgG). ¶, $P < 0.01$ (*vs.* ghrelin + Y1 antagonist + control IgG).

ulates feeding predominantly through Y1 NPY receptor (33, 50). Icv injection of an Y1 NPY receptor antagonist significantly reduced ghrelin-induced food intake. Moreover, coadministration of ghrelin and Y1 NPY receptor antagonist following anti-orexin-A and -B IgGs pretreatment abolished ghrelin-induced feeding. Therefore, ghrelin likely interacts with both the NPY and orexin systems to induce feeding.

Feeding behavior involves the complicated integration of a large number of learning, memory, cognitive, emotional, somatosensorimotor, and autonomic events. Icv administration of ghrelin strongly induces Fos in the hypothalamus, brain stem, hippocampus, dentate gyrus, and piriform cortex (7). Centrally administered ghrelin may regulate feeding and energy homeostasis not only through direct activation of orexin and NPY pathways but also through influences on learning and memory, a state of mood, and formation of emotion. Further investigation of ghrelin interactions with other neuronal systems will provide novel insights into the regulation of feeding and energy homeostasis.

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