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Orexigenic Action of Peripheral Ghrelin Is Mediated by Neuropeptide Y and Agouti-Related Protein

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Ghrelin, a stomach-derived orexigenic hormone, has stimulated great interest as a potential target for obesity control. Pharmacological evidence indicates that ghrelin's effects on food intake are mediated by neuropeptide Y (NPY) and agouti-related protein (AgRP) in the central nervous system. These include intracerebroventricular application of antibodies to neutralize NPY and AgRP, and the application of an NPY Y1 receptor antagonist, which blocks some of the orexigenic effects of ghrelin. Here we describe treatment of *Agrp*$^{-/-}$;*Npy*$^{-/-}$ and *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ double knockout mice as well as *Npy*$^{-/-}$ and *Agrp*$^{-/-}$ single knockout mice with either ghrelin or an orally active nonpeptide ghrelin agonist. The data demonstrate that NPY and AgRP are required for the orexigenic effects of ghrelin, as well as the involvement of the melanocortin pathway in ghrelin signaling. Our results outline a functional interaction between the NPY and AgRP pathways. Although deletion of either NPY or AgRP caused only a modest or nondetectable effect, ablation of both ligands completely abolished the orexigenic action of ghrelin. Our results establish an *in vivo* orexigenic function for NPY and AgRP, mediating the effect of ghrelin. (*Endocrinology* 145: 2607–2612, 2004)

REGULATION OF CALORIC intake and energy expenditure requires precise coordination between peripheral nutrient sensing molecules and central regulatory networks. Stomach-derived ghrelin is the first peripheral orexigenic hormone identified (1–5). Ghrelin was initially found as an endogenous ligand of the GH secretagogue receptor (GHSR). In addition to its ability to stimulate GH secretion, ghrelin can also stimulate caloric intake and increase body weight and adiposity (2–5). Both GH-releasing and the feeding stimulation activities require octanoylation on the serine-3 residue of the 28-amino acid ghrelin (1). Importantly, signaling by circulating ghrelin is mediated downstream by neurons of arcuate nucleus of hypothalamus, in particular, neurons expressing neuropeptide Y (NPY)/agouti-related protein (AgRP) (6–10). NPY and AgRP are two potent orexigenic peptides coexpressed in subsets of neurons in the arcuate nucleus (11). Evidence for the effects of ghrelin on food intake being mediated by NPY and AgRP has been supported by a number of experimental approaches (3, 7–10, 12), including blockade of ghrelin-induced food intake by either intracerebroventricular injection of antibodies against NPY and AgRP (3), NPY Y1 receptor antagonists (3, 12), or peripheral administration of the melanocortin agonist MTII (13). Additionally, electrophysiological approaches have demonstrated that ghrelin can activate NPY/AgRP neurons and simultaneously reduce the activity of proopiomelanocortin (POMC) neurons (14). Because plasma ghrelin levels increase in response to fasting and fall after meal consumption, ghrelin has been proposed to be a short-term feeding initiator (2, 15). Date *et al.* (16) have shown that vagotomy blocks the orexigenic activity of circulating ghrelin, indicating that ghrelin signaling may reach the arcuate nucleus by route of vagal afferents. Alternatively, it has been shown that the blood brain barrier in the arcuate nucleus is deficient and arcuate neurons are exposed to circulating hormones such as leptin (17). Therefore, direct access to the arcuate might also be an important route for ghrelin signaling. The relative contribution of these two routes under various physiological conditions requires further delineation.

To further substantiate the role of AgRP and NPY as mediators of ghrelin's orexigenic actions, we peripherally administered ghrelin or an orally active nonpeptide agonist (compound A) in various strains of double and single knockout mice, including mice lacking both Agrp and NPY (*Agrp*$^{-/-}$; *Npy*$^{-/-}$ mice), mice lacking only NPY (*Npy*$^{-/-}$ mice), mice lacking only Agrp (*Agrp*$^{-/-}$ mice), mice lacking the GH secretagogue receptor (*Ghsr* $^{-/-}$ mice), mice lacking both the melanocortin-3 and -4 receptors (*Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ mice), and wild-type control mice (Refs. 18–20 and *Materials and Methods*). Our results suggest that AgRP and NPY are obligatory mediators of the orexigenic effect of circulating ghrelin and imply that inhibition of melanocortin signaling is required for this effect.

Abbreviations: AgRP, Agouti-related protein; GHSR, GH secretagogue receptor; Mc3r and Mc4r, melanocortin-3 and -4 receptors; NPY, neuropeptide Y; POMC, proopiomelanocortin.

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Materials and Methods

Animals

All animal protocols used in these studies were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee (Rahway, NJ). Mice were housed in microisolator cages (Lab-

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products, Maywood, NJ) on a 12-h light, 12-h dark cycle (lights on at 0700 h).

Agrp$^{-/-}$; *Agrp*$^{-/-}$; *Npy*$^{-/-}$; *Mc3r*$^{-/-}$; *Mc4r*$^{-/-}$, and *Ghsr*$^{-/-}$ mice have been previously described (18–20). The study groups of *Agrp*$^{-/-}$; *Npy*$^{-/-}$ and *Agrp*$^{+/+}$;*Npy*$^{-/-}$ mice were generated by crossing *Agrp*$^{+/-}$ *Npy*$^{+/+}$ mice with *Agrp*$^{+/+}$;*Npy*$^{-/-}$mice. The resulting *Agrp*$^{+/-}$*Npy*$^{+/-}$ mice were backcrossed with *Agrp*$^{+/+}$*Npy*$^{-/-}$ mice to generate *Agrp*$^{+/-}$ *Npy*$^{-/-}$ mice, which were then interbred to produce *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice. At the same time, *Agrp*$^{+/+}$;*Npy*$^{+/-}$mice were crossed with *Agrp*$^{+/+}$; *Npy*$^{+/+}$ mice to generate the *Agrp*$^{+/+}$;*Npy*$^{+/+}$ wild-type control mice with similar genetic background. The genetic background of the *Agrp*$^{-/-}$; *Npy*$^{-/-}$; and *Agrp*$^{+/+}$;*Npy*$^{+/+}$ wild-type mice are 87.5% 129sv; 12.5% C57BL/6. *Npy*$^{-/-}$ single knockout mice are of 100% 129sv background. *Agrp*$^{-/-}$ single knockout and wild-type littermates were generated separately and were of a genetic background of 50% 129sv and 50% C57BL/6. *Mc3r*$^{-/-}$; *Mc4r*$^{-/-}$ double knockout mice were generated by crossing *Mc3r*$^{-/-}$ *with Mc4r*$^{-/-}$ knockout mice, which had been previously backcrossed to C57BL/6J for six generations (98.5% C57BL/6J). *Ghsr*$^{+/+}$, *Ghsr*$^{+/-}$and *Ghsr*$^{-/-}$ littermate mice were generated by intercrossing *Ghsr*$^{+/-}$mice, which had been previously backcrossed to C57BL/6J for three generations (87.5% C57BL/6J/12.5% 129Sv).

Compounds

Native human ghrelin (1–28 with Ser-3 octanoyl group) was synthesized by SynPep Corp. (Dublin, CA). Ghrelin peptidomimetic compound A was prepared by Merck Research Laboratory (21).

In vitro binding and functional assays

Binding assay. Membrane binding assays were performed on transiently transfected COS-7 cells expressing human GH secretagogue receptor (GHSR1a) from the plasmid vector pCI-neo (Promega, Madison, WI) as described (22, 23). Binding buffer contained 25 mM Tris (pH 7.4), 10 mM $MgCl_2$, 2.5 mM EDTA, 0.1% BSA (Sigma, St. Louis, MO), and the following protease inhibitors: 4 g/ml leupeptin (Sigma), 40 g/ml bacitracin (Sigma), 5 g/ml aprotinin (Roche Molecular Biochemicals, Indianapolis, IN), 0.05 M AEBSF (Roche Molecular Biochemicals), and 5 mM phosphoramidon (Roche Molecular Biochemicals). [^{35}S]MK-0677 (0.05 nM, specific activity ~1200 Ci/mmol) or [His[^{125}I]]-human ghrelin (0.1 nM, specific activity ~2000 Ci/mmol; NEN Life Science Products, Boston, MA) was bound to 4 μg of membrane protein/well with or without competing test ligand. The bound membranes were filtered on 0.5% polyethyleneimine prewet filters (UniFilter 96 GF/C, Packard, Meriden, CT). Filters were washed three times [50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 2.5 mM EDTA, 0.05% BSA] dried, and counted with Microscint 20 (Packard, Meriden, CT). Specific binding is defined as the difference between total binding and nonspecific binding conducted in the presence of 500 nM unlabeled human ghrelin. IC_{50} calculations were performed using Prism 3.0 (GraphPad Software, San Diego, CA).

Aequorin bioluminescence assay. A stable cell line expressing the human GH secretagogue receptor (GHSR1a) and the aequorin reporter protein were used to measure agonist-induced mobilization of intracellular calcium as described (22–25).

The binding IC_{50} and functional EC_{50} values were measured in triplicates and representative numbers were given (see Fig. 2).

Rat pituitary GH release. Compound functional activity was evaluated by measuring GH secretion from primary cultures of rat anterior pituitary cells (26). Cells were isolated from rat pituitaries by enzymatic digestion with 250 μg/ml deoxyribonuclease I and 0.5% trypsin in Hanks' balanced salt solution. The cells were suspended in culture medium and adjusted to a concentration of 1.5×10^5 cells/ml, and 1.0 ml of this suspension was placed in each well of a 24-well tray. Cells were maintained in a humidified 5% CO_2/95% air atmosphere at 37 C for 3–4 d. The culture medium consisted of DMEM containing 0.37% $NaHCO_3$, 10% horse serum, 2.5% fetal bovine serum, 1% nonessential amino acids, 1% glutamine, 1% nystatin, and 0.1% gentamicin. Before testing compounds for their capacity to stimulate GH release, cells were washed twice 1.5 h before and once more immediately before the start of the experiment with the above culture medium containing 25 mM HEPES (pH 7.4). Compounds were tested in quadruplicate by adding them in 1 ml of fresh medium to each well and incubating them at 37 C for 15 min followed by centrifugation at 2000 $\times$ *g* for 15 min to remove any cellular material. The supernatant fluid was assayed for GH by a double antibody RIA.

Feeding studies

Mice were individually housed at approximately 1 month of age. Regular mouse chow (Teklad 7012: 13.4% kcal from fat; 3.41 kcal/g, Harlan Teklad, Madison, WI) was provided as pellet food in wire cage tops containing food hoppers. Ghrelin (in 100 μl saline, ip), ghrelin peptidomimetic compound A (in 200 μl aqueous solution containing 5% Tween 80 and 0.5% methylcellulose, taken orally), and corresponding vehicles were administered at 1000 h, and food intake was measured 4 h later. The ages of various study groups were: approximately 13 months for *Agrp*$^{+/+}$;*Npy*$^{-/-}$; *Agrp*$^{-/-}$;*Npy*$^{-/-}$ and *Agrp*$^{+/+}$;*Npy*$^{+/+}$ (average weight = 35 g); 5 months for *Agrp*$^{-/-}$ and wild-type littermates (average weight = 32 g); 9 months for *Mc3r*$^{-/-}$; *Mc4r*$^{-/-}$ (average weight = 55 g) and wild-type littermates (average weight = 28.5 g) and 3 months for *Ghsr*$^{-/-}$ and wild-type littermates (average weight = 27.5 g).

All food intake values were reported as mean ± SEM, and analyzed by the two-tailed, unpaired Student's *t* test. *P* values of < 0.05 were reported as significant.

Results

Considering that ghrelin is produced predominantly by the oxyntic cells located within the fundus of the stomach (27) and its plasma levels fluctuate with meal cycles (15), it is pertinent to evaluate the actions of peripherally administered ghrelin. Our dose titration experiment showed that during light phase, a single ip injection of human ghrelin (10 mg/kg) consistently stimulated 4-h food intake in *ad libitum*-fed wild-type mice. Furthermore, human ghrelin when given to the wild-type mice at 30 or 100 mg/kg significantly increased the 4-h daytime food intake (460 and 500% increases respectively, $P < 0.001$), whereas the same doses had no significant effects on mice deficient for the GH secretagogue receptor (*Ghsr* ko mice, Fig. 1A). These results validate the GH secretagogue receptor as the primary *in vivo* ghrelin receptor responsible for modulating appetite in mice. When administered to *ad libitum*-fed *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice and wild-type control mice, human ghrelin (10 mg/kg) stimulated 4-h food intake by 120% in the wild-type controls but had no effect on food intake by *Agrp;Npy* double knockout mice (Fig. 1B). This result demonstrates that AgRP and NPY are obligatory mediators of the orexigenic effects of circulating ghrelin. Systemic administration of ghrelin has been shown to induce c-fos expression in arcuate nucleus (8, 9) and paraventricular nucleus of hypothalamus (28) and about 90% of the c-fos positive neurons contain NPY. Taken together, these data indicate that peripheral ghrelin acts at least in part through modulation of arcuate NPY/AgRP neurons.

We also evaluated a potent oral ghrelin peptidomimetic, compound A, for its effect on food intake in the wild-type and *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice. Data obtained from an *in vitro* binding assay demonstrated that compound A and ghrelin have comparable binding affinities for the human GH secretagogue receptor (Fig. 2). The mean-effective concentration (EC_{50}) of compound A was 3.1 and 0.7 nM in an intracellular calcium mobilization assay and in an *in vitro* pituitary GH release assay, respectively (Fig. 2). To assess the effect of compound A on food intake, we dosed wild-type mice with the compound by oral gavage. Like ghrelin, compound A

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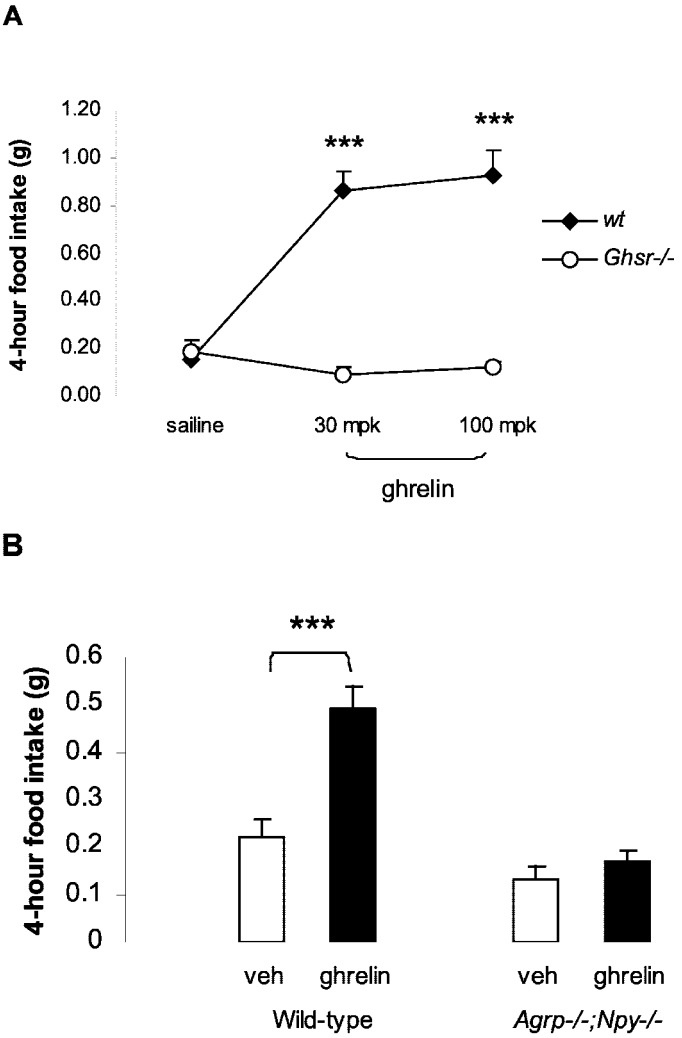
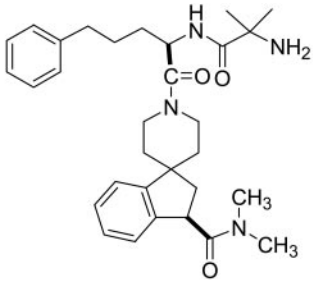


FIG. 1. Orexigenic effects of ghrelin are mediated by the GH secretagogue receptor and are absent in mice deficient for both AgRP and NPY. A, Injection (ip) of ghrelin at doses of 30 mg/kg (mpk) and 100 mg/kg potently stimulated 4-h food intake in wild-type mice (wt; n = 8 for saline; n = 4 for ghrelin; ***, $P < 0.001$ *vs.* saline) but was without effect in GH secretagogue receptor knockout mice (*Ghsr ko*, n = 14 for saline; n = 7 for ghrelin). B, Effect of ghrelin on food intake in $Agrp^{-/-};Npy^{-/-}$ mice (n = 12) and wild-type controls (n = 12). Ghrelin at 10 mg/kg stimulated 4-h food intake in wild-type mice ($P < 0.001$ *vs.* saline) but was without effect in the double knockout mice.

significantly stimulated 4-h food intake in *ad libitum*-fed mice during the daytime, a period of time when they normally would eat very little. The maximum stimulation on feeding was obtained by a dose of 3 mg/kg (Fig. 3A). Compound A was evaluated in the *Ghsr* knockout and wild-type mice at 20 mg/kg (Fig. 3B). The compound administration significantly stimulated 4-h food intake (~725% increase, $P < 0.001$) in the wild-type mice but had no significant effect in the *Ghsr* knockout mice. These results demonstrate that the orexigenic actions of peripherally administered compound A are mediated by GH secretagogue receptor. Interestingly, the heterozygous *Ghsr* mutant mice exhibited a response to compound A, which was intermediate between those observed in wild-type and knockout mice, suggesting a haploinsufficiency phenotype (data not shown).



Compound A

	IC50 or EC50 (nM)	
	Ghrelin	Compound A
^{125}I-human ghrelin binding	0.7	0.3
^{35}S-MK-0677 Binding	0.3	0.1
Aequorin Function	1.4	3.1
Pituitary GH Release	0.7	0.7

FIG. 2. Structure of ghrelin agonist compound A and its *in vitro* binding and functional property compared with human ghrelin.

Figure 4A shows that, when tested as a ghrelin surrogate, a single oral dose of 3 mg/kg of compound A stimulated 4-h daytime food intake in the wild-type control mice by 4-fold when compared with saline (compound A, 0.59 ± 0.06 g *vs.* vehicle, 0.15 ± 0.04 g, $P < 0.001$). The magnitude of stimulation was reduced in $Npy^{-/-}$ mice (compound A, 0.21 ± 0.04 g *vs.* vehicle, 0.01 ± 0.01 g, $P < 0.001$), and compound A was without effect on the *Agrp;Npy* double-null mice. A separate group of *Agrp* single knockout mice and their wild-type littermates were also tested by oral dosing of 3 mg/kg compound A. The compound was equally efficacious in the *Agrp* single knockout mice as in the wild-type controls (Fig. 4B). These data indicate that removal of NPY severely compromised the feeding promotion of ghrelin agonist compound A, whereas the loss of AgRP does not by itself diminish ghrelin signaling. The loss of NPY activity can be partially compensated for by AgRP as shown by feeding stimulation in the NPY single knockout mice. The removal of both NPY and AgRP, however, completely abolished signaling by ghrelin or the ghrelin agonist compound A.

Our results are consistent with pharmacological studies indicating that antibodies directed against NPY or NPY receptor Y1 antagonists could attenuate ghrelin-induced food intake (3, 12). Additionally, we showed that $Agrp^{-/-}$ mice responded to ghrelin agonist compound A to a similar extent as the wild-type mice during the specified period of observation. This is in contrast to previous results demonstrating that intracerebroventricular administration of an antibody directed against AgRP can attenuate ghrelin-induced feeding (3). This suggests that either a developmental compensation took place, allowing the $Agrp^{-/-}$ mice to respond to ghrelin, or that feeding inhibition by the anti-AgRP antibody resulted from a nonspecific effect.

NPY stimulates food intake predominantly through acti-

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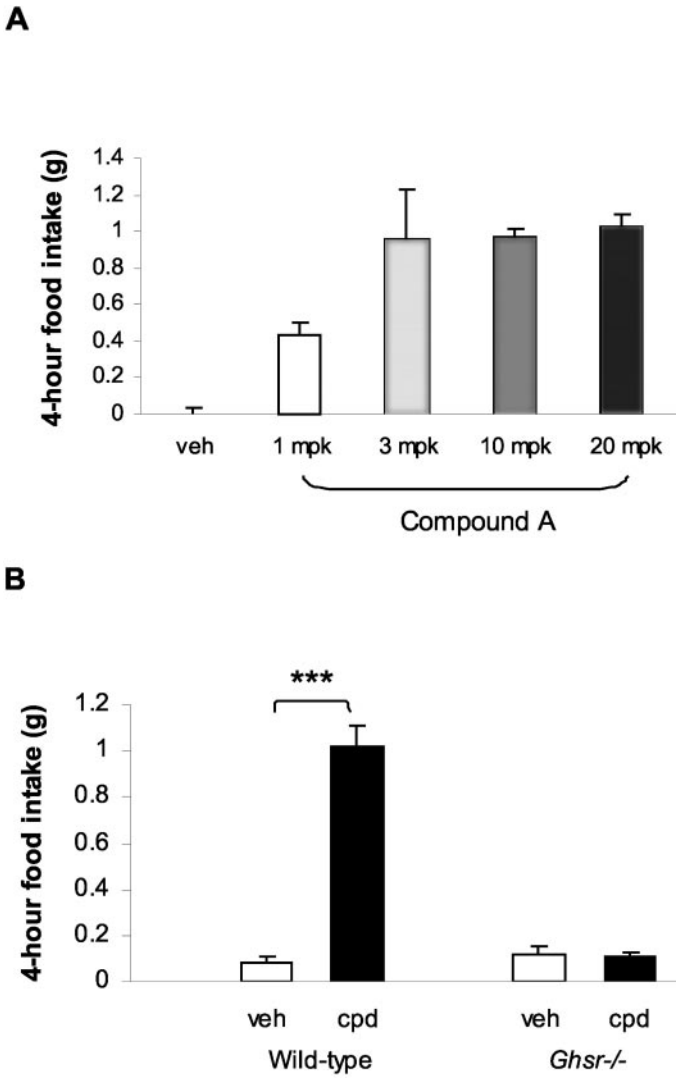

FIG. 3. Effect of ghrelin agonist compound A on food intake in C57BL/6 and GH secretagogue receptor-deficient mice. A, All four doses (oral dosing) of compound A significantly stimulated 4-h food intake ($P < 0.01$ *vs.* vehicle) in C57BL/6 mice. The maximum effect was reached by 3 mg/kg (mpk). B, Compound A at 20 mg/kg stimulated 4-h food intake in wild-type controls (***, $P < 0.001$ *vs.* vehicle) but was without effect in GH secretagogue receptor knockout mice ($Ghsr^{-/-}$).

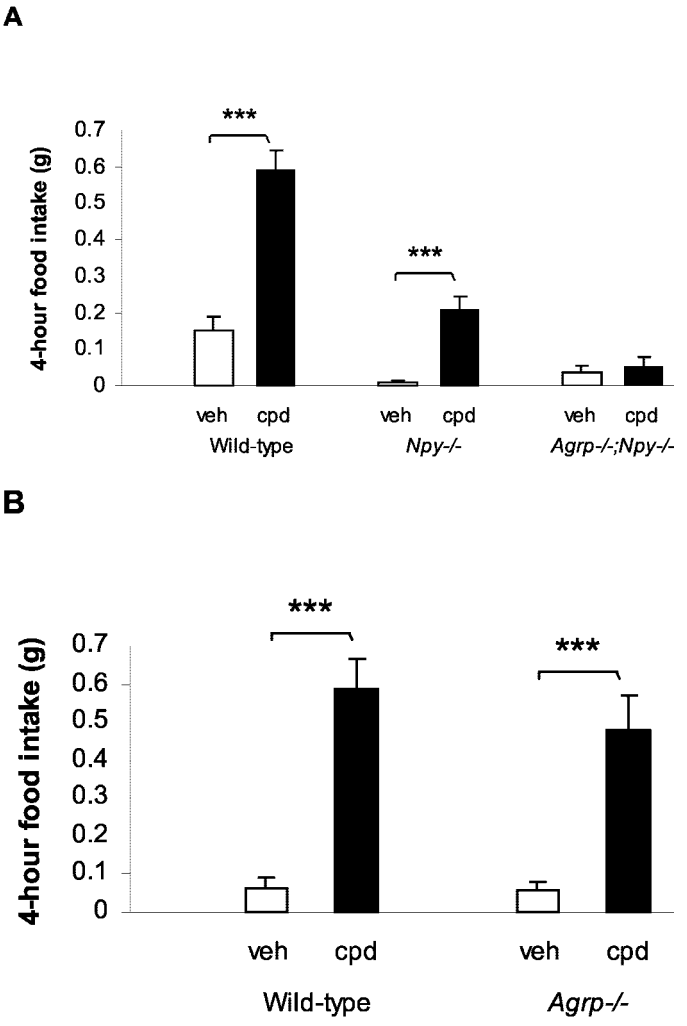

FIG. 4. Effect of ghrelin agonist compound A (3 mg/kg, taken orally) on food intake in *Agrp* single knockout, *Npy* single knockout, and *Agrp;Npy* double knockout mice. A, Compound A significantly stimulated 4-h food intake in wild-type mice (n = 12; ***, $P < 0.001$ *vs.* saline). The compound stimulated food intake in Npy single knockout mice ($Npy^{-/-}$; n = 12), but the effect was reduced ($P < 0.001$ *vs.* saline). The compound did not stimulate food intake in the *Agrp;Npy* double null mice ($Agrp^{-/-};Npy^{-/-}$, n = 12). B, Compound A significantly stimulated 4-h food intake in wild-type (n = 16; $P < 0.001$ *vs.* saline) and *Agrp* single knockout mice ($Agrp^{-/-}$; n = 16; $P < 0.001$ *vs.* saline).

vation of NPY Y1 and Y5 receptors and both AgRP and NPY stimulate food intake by inhibiting arcuate POMC neurons and hypothalamic melanocortin tone (24, 27, 29, 30). Central melanocortin signaling affecting energy homeostasis is mediated mostly by melanocortin receptor 4 (Mc4r), and to a lesser degree by melanocortin receptor 3 (Mc3r) (18, 31–33). To determine to what extent ghrelin signaling is mediated by the melanocortin system, we evaluated the orexigenic potential of ghrelin and the ghrelin agonist compound A on the mutant mice deficient in both Mc3r and 4r (*Mc3r;Mc4r* double knockout mice). Wild-type control mice increased daytime food consumption in response to both ghrelin and compound A (Fig. 5). Food intake of the *Mc3r;Mc4r* double knockout mice tended to be higher in response to ip ghrelin, but the increase did not reach statistical significance (Fig. 5A). The *Mc3r;Mc4r* double null mice responded to orally dosed compound A with a significant increase in food intake (compound A, 0.45 ± 0.08 g *vs.* vehicle, 0.19 ± 0.08 g, $P < 0.05$, Fig. 5B). However, the magnitude of the increase was much less than that observed in wild-type mice (compound A, 0.88 ± 0.08 g *vs.* vehicle, 0.18 ± 0.07 g, $P < 0.001$, Fig. 5B). The compound A stimulated 4-h food intake in the *Mc3r;Mc4r* double null mice was statistically different from that in the wild-type controls (0.45 + 0.08 *vs.* 0.88 + 0.08 g, $P < 0.01$). The compound A appears to be more potent than ghrelin in mice. This likely results from its greater stability *in vivo*, not due to a different mode of action as both agents were inactive in *Ghsr*-deficient mice. These data suggest that AgRP and NPY inhibition of melanocortin signaling is the principal

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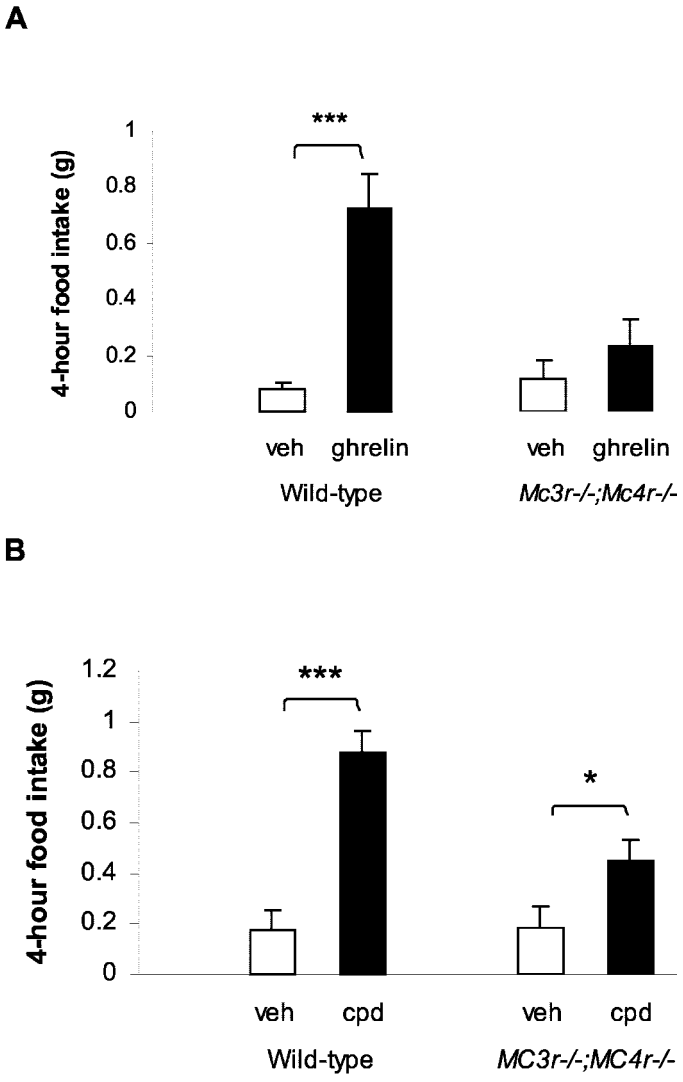

FIG. 5. Effects of ghrelin and compound A in *Mc3r;Mc4r* double knockout mice. A, Ghrelin (10 mg/kg, ip) stimulated 4-h food intake in wild-type mice (n = 9; ***, $P < 0.001$ *vs.* saline) but was without effect in the $Mc3r^{-/-};Mc4r^{-/-}$ mice (n = 12). B, Compound A (3 mg/kg, taken orally) stimulated 4-h food intake in wild-type mice (n = 9, $P < 0.001$ *vs.* vehicle). The feeding stimulation was attenuated in the $Mc3r^{-/-};Mc4r^{-/-}$ mice (n = 12; *, $P < 0.05$ *vs.* vehicle).

mechanism responsible for the orexigenic effect of circulating ghrelin, but because the *Mc3r;Mc4r* double null mice still partially responded to compound A, a significant amount of ghrelin action is mediated through nonmelanocortin pathways.

Discussion

In this report, we demonstrated that the GH secretagogue receptor is the primary *in vivo* ghrelin receptor responsible for modulating appetite in mice. The evaluation in the *Ghsr*-deficient mice helped identify the orally available compound A as a potent small molecule ghrelin mimetic. The lack of orexigenic effect in *Ghsr*-deficient mice argues that both ghrelin and compound A stimulate feeding by the same mechanism of action. When compared in wild-type C57BL6 mice, compound A at the maximum effective dose of 3 mg/kg confers greater feeding stimulation than 10 mg/kg ghrelin. This apparent greater efficacy *in vivo* can be partially explained by the lower molecular weight of compound A (molecular weight = 555), compared with ghrelin (molecular weight = 3369). However, being a nonpeptide small molecule, compound A could also have better pharmacokinetic properties, such as longer half-life and greater penetration of blood brain barrier. These attributes may also contribute to its greater efficacy *in vivo*, making it a useful tool for analysis of the ghrelin signaling pathways.

The lack of response of the *Agrp;Npy* double deficiency mice to feeding stimulation by ghrelin and the ghrelin agonist compound A agrees well with the results of pharmacological studies described previously and validates that AgRP and NPY are physiological orexigenic factors. One principal function of the two neuropeptides appears to be communicating between the peripheral hunger hormone ghrelin and the central melanocortin circuit. The *Agrp;Npy* double knockout mice have normal growth rates and do not suffer from a feeding deficit under free feeding conditions or after fasting. It was proposed that other orexigenic pathways might compensate for the deficiency of AgRP and NPY (18). The importance of AgRP and NPY in mediating ghrelin signaling suggests that the two neuropeptides are involved in the regulation of feeding initiation. Under laboratory conditions where food is constantly available, the deficit in feeding initiation may not necessarily reduce overall caloric intake, as the mice can increase food consumption in later portions of the feeding period. This interpretation is consistent with the results that physiological doses of peripheral ghrelin only stimulate short-term food intake in mice (4, 5, 10). In a food-scarce environment, however, animals that initiate feeding more slowly might be at a competitive disadvantage.

The attenuation of ghrelin effect in the *Mc3r;Mc4r* double knockout mice revealed that ghrelin stimulates energy intake in part by suppressing hypothalamic melanocortin tone. Immunohistochemistry and electrophysiology studies have shown that NPY neurons synapse on and inhibit POMC neurons directly (31). NPY also activates inhibitory GABAergic interneurons that innervate neurons expressing POMC and MC4r (24), thereby inhibiting melanocortin signaling indirectly. Our data are consistent with the antagonistic interaction between the NPY/AgRP neurons and melanocortin neurons. Ghrelin activation of arcuate NYP/AgRP neurons, either by route of vagal afferents or blood circulation, will lead to inhibition of melanocortin signaling both directly and indirectly, resulting in an increase of food intake. The fact that compound A is inactive in the *Agrp;Npy* double knockout mice but partially active in the *Mc3r;Mc4r* double knockout mice, however, indicates that NPY effects are not limited to inhibiting the melanocortin pathway

In summary, we presented evidence that that feeding stimulation by peripheral ghrelin acts via NPY and AgRP, with NPY as a primary effector. These results thus clarify that one of the *in vivo* functions of NPY and AgRP is to relay peripheral ghrelin signaling. We also showed that ghrelin signaling depends on the integrity of central melanocortin system, whereas others have shown it is also dependent on the orexin

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pathway (34). Recently, low levels of ghrelin expression were detected in the periventricular areas between major hypothalamic nuclei (14). The ghrelin expressing neurons project axon terminals to innervate important hypothalamic neurons involved in feeding regulation, including NPY and POMC neurons. It would be interesting to investigate if the hypothalamic ghrelin level fluctuates in response to nutritional status and if central ghrelin is part of the integrated hunger sensing system.

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