

Ghrelin Causes Hyperphagia and Obesity in Rats

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Ghrelin, a circulating growth hormone-releasing peptide derived from the stomach, stimulates food intake. The lowest systemically effective orexigenic dose of ghrelin was investigated and the resulting plasma ghrelin concentration was compared with that during fasting. The lowest dose of ghrelin that produced a significant stimulation of feeding after intraperitoneal injection was 1 nmol. The plasma ghrelin concentration after intraperitoneal injection of 1 nmol of ghrelin (2.83 ± 0.13 pmol/ml at 60 min postinjection) was not significantly different from that occurring after a 24-h fast (2.79 ± 0.32 pmol/ml). After microinjection into defined hypothalamic sites, ghrelin (30 pmol) stimulated food intake most markedly in the arcuate nucleus (Arc) (0–1 h food intake, $427 \pm 43\%$ of control; $P < 0.001$ vs. control, $P < 0.01$ vs. all other nuclei), which is potentially accessible to the circulation. After chronic systemic or intracerebroventricular (ICV) administration of ghrelin for 7 days, cumulative food intake was increased (intraperitoneal ghrelin 13.6 ± 3.4 g greater than saline-treated, $P < 0.01$; ICV ghrelin 19.6 ± 5.5 g greater than saline-treated, $P < 0.05$). This was associated with excess weight gain (intraperitoneal ghrelin 21.7 ± 1.4 g vs. saline 10.6 ± 1.9 g, $P < 0.001$; ICV ghrelin 15.3 ± 4.3 g vs. saline 2.2 ± 3.8 g, $P < 0.05$) and adiposity. These data provide evidence that ghrelin is important in long-term control of food intake and body weight and that circulating ghrelin at fasting concentrations may stimulate food intake. *Diabetes* 50: 2540–2547, 2001

A range of synthetic growth hormone secretagogues (GHSs) act at the growth hormone secretagogue receptor (GHS-R) to stimulate secretion of growth hormone (GH) in several species, including humans (1,2). The GHS-R is expressed in discrete hypothalamic nuclei, which have been implicated in body weight regulation, notably in the Arc, the paraventricular nucleus (PVN), and the ventromedial nu-

cleus (VMN) (3–5). During the development of synthetic GHSs, weight gain was noted after chronic systemic administration in immature rodents (1). Ghrelin is a circulating 28-amino acid peptide that was recently purified from rat stomach, and the gene was subsequently cloned in rats and humans (6). It is the first identified endogenous ligand for the GHS-R and is highly conserved across species, differing by only two amino acids between rat and human (6).

Ghrelin is synthesized primarily in X/A-like endocrine cells in the oxyntic glands of the stomach and is present in the circulation (7). Circulating ghrelin is elevated after a 48-h fast and subsequently lowered by 50% glucose administration into the stomach but not by the same volume of water (8). Ghrelin is found at lower levels in the hypothalamus, where ghrelin immunoreactivity is confined to the arcuate nucleus of colchicine-treated rats (6). The Arc is an important site in the control of food intake (9). The potent orexigenic neurotransmitters neuropeptide Y (NPY) and Agouti-related protein (AgRP) are colocalized in neurons in the medial Arc (10). After systemic administration of ghrelin or GHSs, *c-fos*-like immunoreactivity (FLI), an indicator of neuronal activation, is evident only in the Arc (11,12). A proportion of these FLI-positive cells are NPY/AgRP neurons (12), which have been shown to express the GHS-R (13). Thus, arcuate neurons that produce well-characterized orexigenic signals are potential targets for circulating ghrelin.

The mechanisms that determine normal body weight regulation are not fully understood but are thought to involve hypothalamic neuronal systems responsive to peripheral signals of nutritional status. Leptin is a well-characterized satiety signal derived from adipose tissue, which acts on hypothalamic neurons, particularly those in the Arc (14,15). By analogy, ghrelin, released from the stomach in response to fasting, may act as a counter-regulatory orexigenic signal to the hypothalamus. Ghrelin has been shown to stimulate food intake after acute systemic (intraperitoneal) or intracerebroventricular (ICV) administration (8,16). The systemic doses of ghrelin used in these studies resulted in plasma ghrelin concentrations much higher than those seen physiologically (8). It is not known how relevant this potent pharmacological stimulation of feeding is to the physiological regulation of food intake.

We aimed to establish whether systemic administration of low-dose ghrelin, resulting in plasma ghrelin levels similar to those that occur during fasting, would stimulate feeding. The lowest dose of ghrelin to significantly stimulate feeding after intraperitoneal injection was investi-

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AgRP, Agouti-related protein; AHA, anterior hypothalamic area; Arc, arcuate nucleus; CNS, central nervous system; DMN, dorsomedial nucleus; FLI, *c-fos*-like immunoreactivity; GH, growth hormone; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; ICV, intracerebroventricular; IGF-1, insulin-like growth factor-1; NPY, neuropeptide Y; PVN, paraventricular nucleus; T4, thyroxine; TSH, thyroid-stimulating hormone; VMN, ventromedial nucleus.

TABLE 1—Coordinates of hypothalamic nuclei used for cannulation

Targeted area	Posterior from bregma (mm)	Lateral from midline (mm)	Depth (mm)
MPO	0.8	0.3	8.8
SON	1.3	1.8	9.3
AHA	1.8	0.6	8.8
PVN	1.8	0.5	8.0
VMN	2.8	0.7	10.0
DMN	3.1	0.5	8.8
ARC	3.3	0.3	10.0
LHA	3.6	1.7	8.8

MPO, medial preoptic area; SON, supraoptic nucleus; LHA, lateral hypothalamic area.

gated, and the resulting circulating ghrelin concentration was compared with that seen during fasting. The Arc is a likely target for circulating ghrelin, but the GHS-R is also expressed in other discrete hypothalamic sites. To investigate which nuclei are involved in the feeding response, we measured food intake in response to microinjection of ghrelin into defined hypothalamic sites. Finally, to assess the possible role of ghrelin in long-term body weight control, we also examined the effect of chronic ghrelin administration on food intake and body weight and composition.

RESEARCH DESIGN AND METHODS

Animals. Male Wistar rats (250–300 g) were maintained in individual cages under controlled temperature (21–23°C) and light (12 h light, 12 h dark, lights on at 0700 h) with ad libitum access to food (RM1 diet; SDS UK) and water. All animal procedures undertaken were approved by the British Home Office Animals Scientific Procedures Act 1986 (Project license no. 90/1077).

Intraperitoneal injections. Rats were accustomed to intraperitoneal injection by sham injections of 0.5 ml saline 2 days before study. For all studies, rats received an intraperitoneal injection of ghrelin or saline in 0.5-ml volume.

Intranuclear and ICV cannulation and injection. Animal surgical procedures and handling were carried out as previously described (17,18). Animals were anesthetized by intraperitoneal injection of a mixture of ketamine (Ketalar HCl 60 mg/kg; Parke-Davis, Pontypool, UK) and xylazine (Rompun 12 mg/kg; Bayer UK, Bury St. Edmunds, UK) and placed in a Kopf stereotaxic frame. For intranuclear cannulation, permanent 26-gauge stainless steel guide cannulae were implanted in the animals (Plastics One, Roanoke, VA) projecting into the Arc, PVN, VMN, medial preoptic area, supraoptic nucleus, anterior hypothalamic area (AHA), dorsomedial nucleus (DMN), and lateral hypothalamic area of the hypothalamus, according to coordinates of Paxinos and Watson (19) (Table 1, Fig. 1). For ICV cannulation, permanent 22-gauge stainless steel guide cannulae were placed into the third cerebral ventricle (0.8 mm posterior to the bregma on the mid-sagittal line 6.5 mm below the outer surface of the skull, coordinates calculated using atlas of Paxinos and Watson [19]). All compounds were dissolved in 0.9% saline, and each study involved an injection of ghrelin or saline in a volume of 1 μ l (for intranuclear studies) or 5 μ l (for ICV studies) over 1 min. Substances were administered by a 31-gauge (for intranuclear studies) or a 27-gauge (for ICV studies) stainless steel injector placed in and projecting 1 mm below the tip of the cannulae. All substances were administered in the early light phase (0900–1000 h). Correct intranuclear cannula placement was confirmed histologically at the end of the study period. After injection of 1 μ l black ink, animals were decapitated, and the brains were removed and immediately frozen in liquid nitrogen and stored at -70°C . Brains were sliced on a cryostat (Bright, Huntingdon, UK) into 15 μm coronal sections and stained with cresyl violet. Correct ICV cannula placement was confirmed by a positive dipsogenic response to angiotensin II (150 ng/rat). Only those animals with correct placement of cannulae were included in the data analysis.

Study 1: determination of whether systemic ghrelin stimulates feeding in satiated rats at the plasma levels found during fasting. The lowest intraperitoneal dose of ghrelin that significantly stimulated feeding in freely fed rats was examined. Rats ($n = 9$ –11 per group) received intraperitoneal injections of saline or ghrelin (30 pmol, 100 pmol, 300 pmol, 1 nmol, 3 nmol, or 10 nmol) at 0800–900 h. Immediately after injection, rats were returned to

their home cages, which contained a preweighed amount of food. The remaining food in the hopper was reweighed at 1, 2, 4, and 24 h postinjection using an ATP Instrumentation GW 600 balance (ATP Instrumentations, Leicestershire, U.K.) recording to the nearest 0.1 g. For determining ghrelin plasma concentration, after a 72-h recovery, rats ($n = 9$ –11 per group) then received an intraperitoneal injection of either saline or ghrelin (1 nmol) at 0900–1000 h. Animals were decapitated 15, 60, or 120 min postinjection. Trunk blood was collected into plastic lithium heparin tubes containing 0.6 mg aprotinin (Bayer, Haywards Heath, UK). Plasma was separated by centrifugation, frozen, and stored at -70°C until radioimmunoassay. An additional group of rats ($n = 10$ per group) were either fed freely or fasted for 24 h then killed by decapitation at 1000–1100 h, and plasma was collected as above.

Study 2: localization of the hypothalamic site of the orexigenic action of ghrelin. Pilot studies suggested that the Arc might be particularly sensitive to ghrelin. An intra-arcuate dose-response study was first performed. Intra-arcuate cannulated rats ($n = 7$ –10 per group) received saline or ghrelin (1, 10, 30, or 100 pmol) at 0900–1000 h, and food intake was measured as above. The lowest dose to stimulate feeding significantly after injection into the Arc was 30 pmol. This dose, therefore, was used to compare the potency of the orexigenic action of ghrelin between each of the hypothalamic nuclei, which was the primary aim of this study. In addition, we wished to examine whether nuclei that failed to respond to 30 pmol of ghrelin would respond at a higher ghrelin concentration. For this purpose, a 10-fold higher dose of 300 pmol was used to provide an excess of ghrelin. Nuclei that failed to respond to this

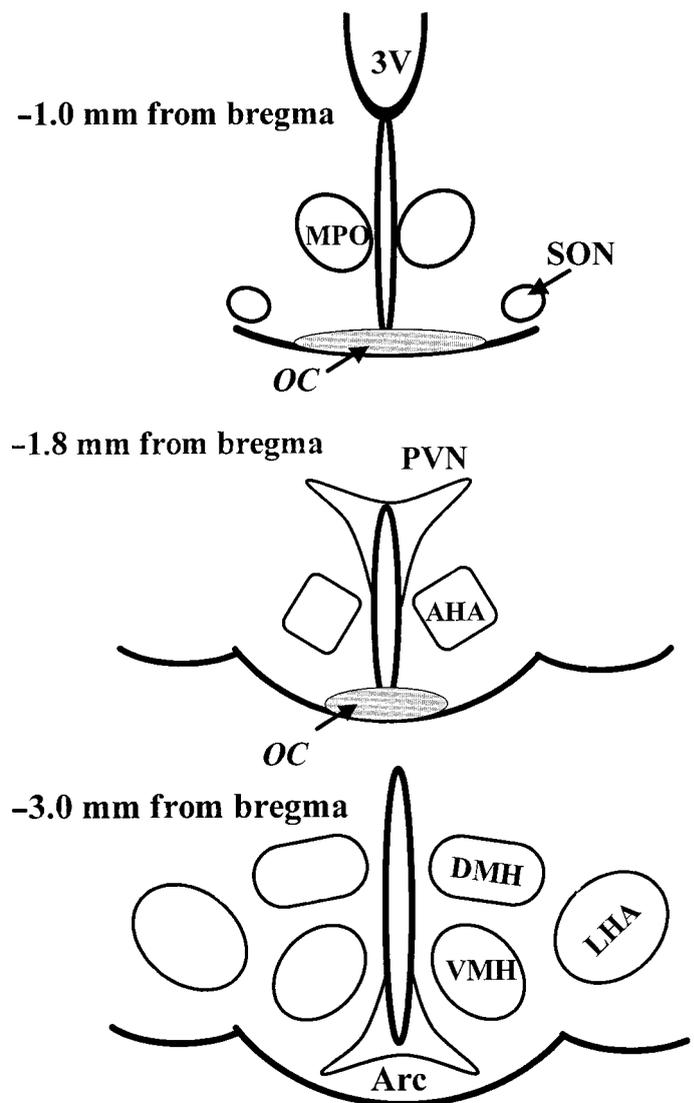


FIG. 1. Schematic representation of coronal sections through the rat hypothalamus, depicting the relationships of the hypothalamic nuclei cannulated in this study, the third ventricle (3V), and the optic chiasm (OC).

higher dose of ghrelin could be considered to be nonresponsive. The study was of randomized crossover design. All animals ($n = 12\text{--}15/\text{group}$) received saline and 30 and 300 pmol of ghrelin by intranuclear microinjection. Animals received the injection in the early light phase (0900–1000 h), and food intake was measured as above.

Study 3: investigation of the effect of repeated intraperitoneal administration of ghrelin on food intake and body weight. Freely fed rats received an intraperitoneal injection of either saline ($n = 15$) or ghrelin (10 nmol, $n = 10$) three times daily for 7 days during the light phase (at 0800 h, 1200 h, and 1600 h). The dose of ghrelin administered was the lowest dose to give a maximum feeding response in study 1 and in previously published data (16). The interval was chosen to space injections throughout the light phase to stimulate feeding in satiated rats, which normally feed nocturnally, and to avoid possible tachyphylaxis as reported for the GH response to more frequent administration of GHs (20). Body weight was measured daily at 0800 h. Food was weighed at the time of each injection and at 1 h after each injection. This allowed calculation of cumulative food intake and food intake at 1 h in response to each injection. A final food and body weight measurement was taken at 0800 h on day 8.

Study 4: investigation of the effect of repeated ICV administration of ghrelin on food intake and body weight. Rats received an ICV injection of either saline ($n = 20$) or 3 nmol ghrelin ($n = 18$) once daily for 7 days during the early light phase (0900 h). Ghrelin 3 nmol was chosen as the lowest dose previously demonstrated to increase 24-h food intake (16). Food and body weight were determined daily just before injection and at 0900 h on the day after the final injection. Food was also reweighed at 1 h after each injection.

Study 5: investigation of the effect of repeated ghrelin administration on body composition and plasma hormones. Rats from studies 3 and 4 were killed by decapitation on day 8 at 0900–1000 h, and plasma was collected as above. The weights of kidney and spleen (to assess for organomegaly) and epididymal fat pad were determined.

Radioimmunoassays. Plasma GH and thyroid-stimulating hormone (TSH) were assayed using reagents and methods provided by the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor University of California, Los Angeles Medical Center) as previously described (17). Plasma ghrelin, total thyroxine (T₄), and insulin-like growth factor-1 (IGF-1) were measured using commercially available radioimmunoassay kits (Phoenix Pharmaceuticals; Coat-a-Count Total T₄, Diagnostic Products; and Diagnostic Systems Laboratories, respectively).

Statistical analysis. Results are shown as means \pm SE, unless otherwise specified. Unpaired Student's *t* test was used for comparisons between two unpaired treatment groups. One-way analysis of variance with post hoc least significant differences test (Systat, Evanston, IL) was used for comparisons between three or more unpaired treatment groups, i.e., the feeding dose responses to intraperitoneal and intra-arcuate ghrelin and the plasma ghrelin concentrations after ghrelin injection. $P < 0.05$ was considered to be statistically significant. For intranuclear studies, paired Student's *t* test was used for comparisons of each dose of ghrelin with saline within a hypothalamic nucleus at each time point. One-way analysis of variance with post hoc Tukey's test was used for comparisons of the effect of ghrelin between nuclei. In view of the large number of comparisons, at least $P < 0.01$ was required for statistical significance for the intranuclear data.

RESULTS

Study 1: determination of whether ghrelin stimulates feeding in satiated rats at the plasma levels found during fasting.

We showed previously that intraperitoneal administration of ghrelin (3, 10, or 30 nmol) stimulates food intake dose-dependently. In the present study, the lowest intraperitoneal dose of ghrelin that caused significant stimulation of food intake was 1 nmol (0–2 h food intake after 1 nmol ghrelin 1.2 ± 0.2 g vs. saline 0.5 ± 0.2 g; $P < 0.05$), although there was a nonsignificant trend toward increase in food intake with doses as low as 30 pmol (Fig. 2A). To assess the physiological relevance of feeding stimulation by intraperitoneal ghrelin, we compared the plasma levels of ghrelin after administration of 1 nmol ghrelin i.p. with those that occurred during fasting. Plasma ghrelin was significantly elevated after a 24-h fast in male Wistar rats (2.79 ± 0.32 vs. fed rats 1.28 ± 0.12 pmol/ml; $P < 0.001$) (Fig. 2B). The plasma ghrelin concen-

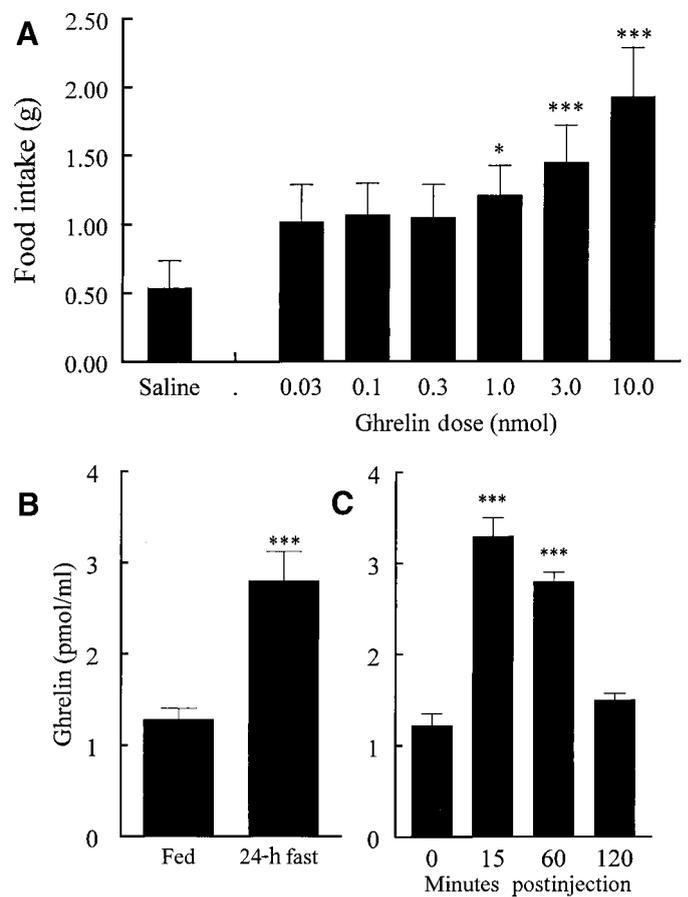


FIG. 2. *a*: Food intake 0–2 h after intraperitoneal injection of saline or ghrelin 0.03–10 nmol; * $P < 0.05$, *** $P < 0.001$ versus saline control. Comparison of plasma ghrelin concentration after 24-h fast (*b*) or intraperitoneal injection (*c*) of 1 nmol of ghrelin in freely fed rats, *** $P < 0.001$ versus freely fed rats and controls that did not receive an injection, respectively.

tration after injection of 1 nmol of ghrelin ip in fed rats was measured up to 2 h (the duration of the feeding response) postinjection. The ghrelin concentrations achieved were not significantly greater than those that occurred after a 24-h fast (Fig. 2C) ($t = 0$, 1.22 ± 0.13 ; $t = 15$ min, 3.30 ± 0.23 ; $t = 60$ min, 2.83 ± 0.12 ; $t = 120$ min, 1.52 ± 0.07 pmol/ml).

Study 2: localization of the hypothalamic site of the orexigenic action of ghrelin.

A feeding dose response was performed to determine the doses of ghrelin to be used for intranuclear comparisons. The lowest dose of ghrelin to significantly stimulate feeding was 30 pmol (0–1 h food intake 2.0 ± 0.4 g vs. saline-treated 0.7 ± 0.3 g; $P < 0.05$) (Fig. 3). This dose of ghrelin, therefore, was used to compare the orexigenic response between hypothalamic nuclei. To examine whether nuclei that failed to respond to 30 pmol ghrelin would respond at a higher ghrelin concentration, we also used a dose of 300 pmol. The results are expressed as percentage of control, as these are paired data observations with each animal receiving each test substance. The orexigenic response to ghrelin after intranuclear injection was greatest at 0–1 h postinjection. At 0–1 h after injection of 30 pmol ghrelin, food intake was significantly greater in animals cannulated into the Arc than into all of the other nuclei studied (Fig. 4) ($427 \pm 40\%$ control; $P < 0.001$ vs. control, $P < 0.01$ vs. PVN, $P < 0.001$

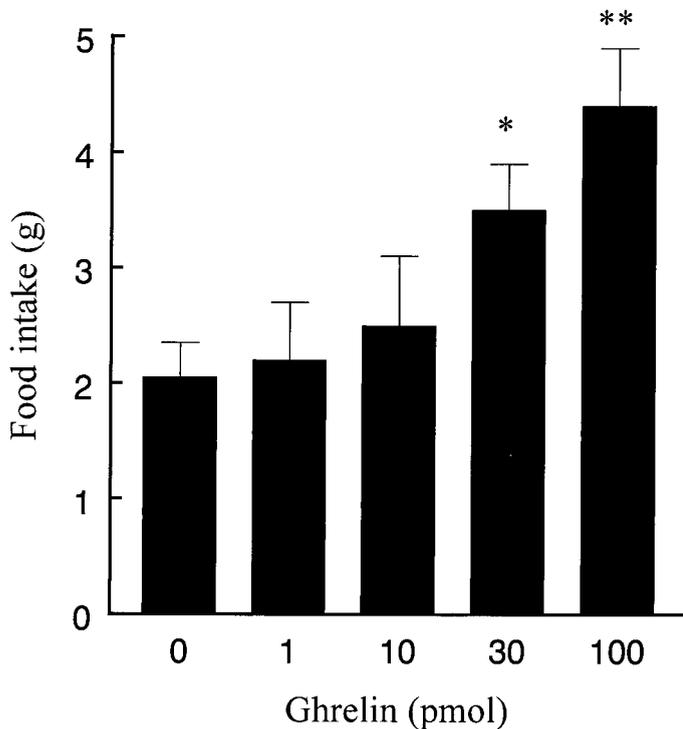


FIG. 3. Food intake at 1 h postinjection of saline or ghrelin (1–100 pmol) into the Arc. * $P < 0.05$, ** $P < 0.01$ versus saline.

vs. all other nuclei). Ghrelin 30 pmol also significantly stimulated feeding at 0–1 h after injection into the PVN ($243 \pm 43\%$, $P < 0.01$) (Fig. 4) but of a lesser magnitude than that observed in the Arc. The effect on food intake up to 24 h postinjection of both doses of ghrelin into all nuclei is shown in Fig. 5.

The low (30 pmol) dose of ghrelin significantly stimulated feeding in only two other nuclei: the VMN and the DMN. The feeding response, however, was delayed and was not significant until 1–2 h postinjection (Fig. 5). In contrast, the high dose of ghrelin (300 pmol) significantly stimulated feeding at 0–1 h in all nuclei. This stimulation was sustained up to 2 h in the PVN, VMN, DMN, and AHA. The orexigenic response after injection of all nuclei was

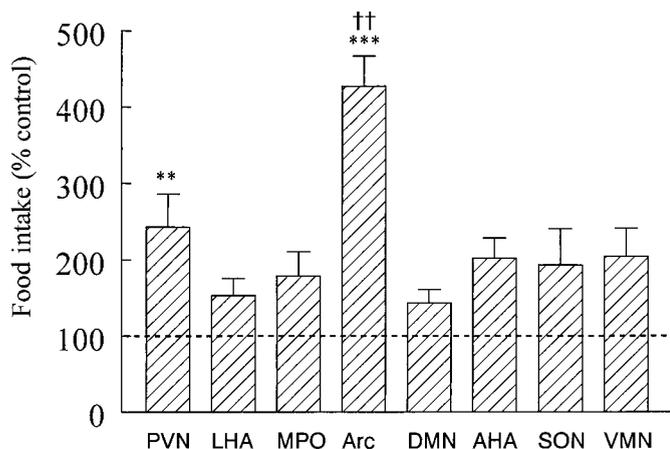


FIG. 4. Comparison between hypothalamic nuclei of food intake stimulated by 30 pmol of ghrelin at 0–1 h postinjection. Food intake is expressed as a percentage of control. Control food intake is indicated by dotted line. †† $P < 0.01$ versus PVN, $P < 0.001$ versus all other nuclei; *** $P < 0.001$ versus saline, ** $P < 0.01$ versus saline.

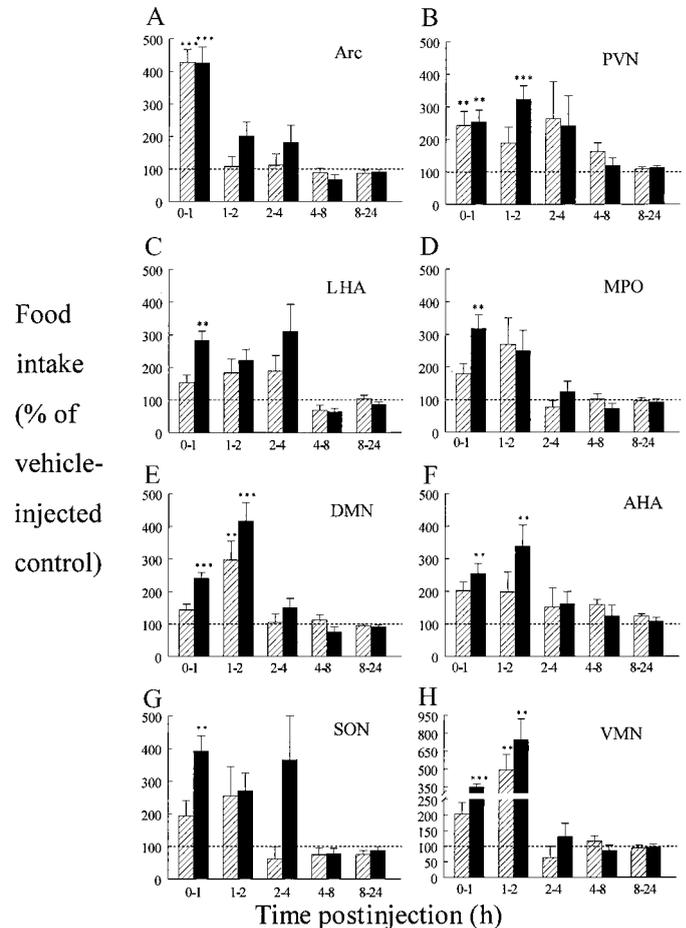


FIG. 5. Effect on food intake of injection of 30 pmol (▨) or 300 pmol ghrelin (■) into Arc (A), PVN (B), lateral hypothalamic area (C), medial preoptic area (D), DMN (E), AHA (F), supraoptic nucleus (G), and VMN (H) over a 24-h period in freely fed rats. Food intake is expressed as a percentage of saline control with control food intake (100%) indicated by dotted line. ** $P < 0.01$, *** $P < 0.001$ versus saline.

short-lived, with no significant stimulation of feeding observed between 2 and 24 h postinjection.

The stimulation of feeding at 1–2 h after injection into the VMN initially seems greater than that observed at 0–1 h after injection of the Arc, when expressed as a percentage of control. However, this is due to the small amount of food eaten by the saline-treated groups at 1–2 h. The absolute stimulation of food intake (grams per rat) compared with control animals was much greater at 0–1 h after arcuate injection (30 pmol ghrelin, 2.4 ± 0.22 g/rat; 300 pmol of ghrelin, 2.4 ± 0.27 g/rat; vs. saline-treated, 0.6 ± 0.24 g/rat) than after VMN injection at 1–2 h (30 pmol of ghrelin, 0.8 ± 0.19 g/rat; 300 pmol of ghrelin, 1.2 ± 0.28 g/rat; vs. saline, 0.2 ± 0.1 g/rat).

Study 3: investigation of the effect of repeated intraperitoneal administration of ghrelin on food intake and body weight. Systemic (intraperitoneal) injection of 10 nmol ghrelin increased cumulative food intake when administered three times daily for 7 days (Fig. 6A). There was no apparent attenuation of ghrelin-induced feeding on repeated administration. Ghrelin administration increased cumulative food intake by stimulating feeding in satiated rats in the light phase, when food intake is usually minimal. There was no significant difference in nocturnal food intake between ghrelin- and saline-treated animals on

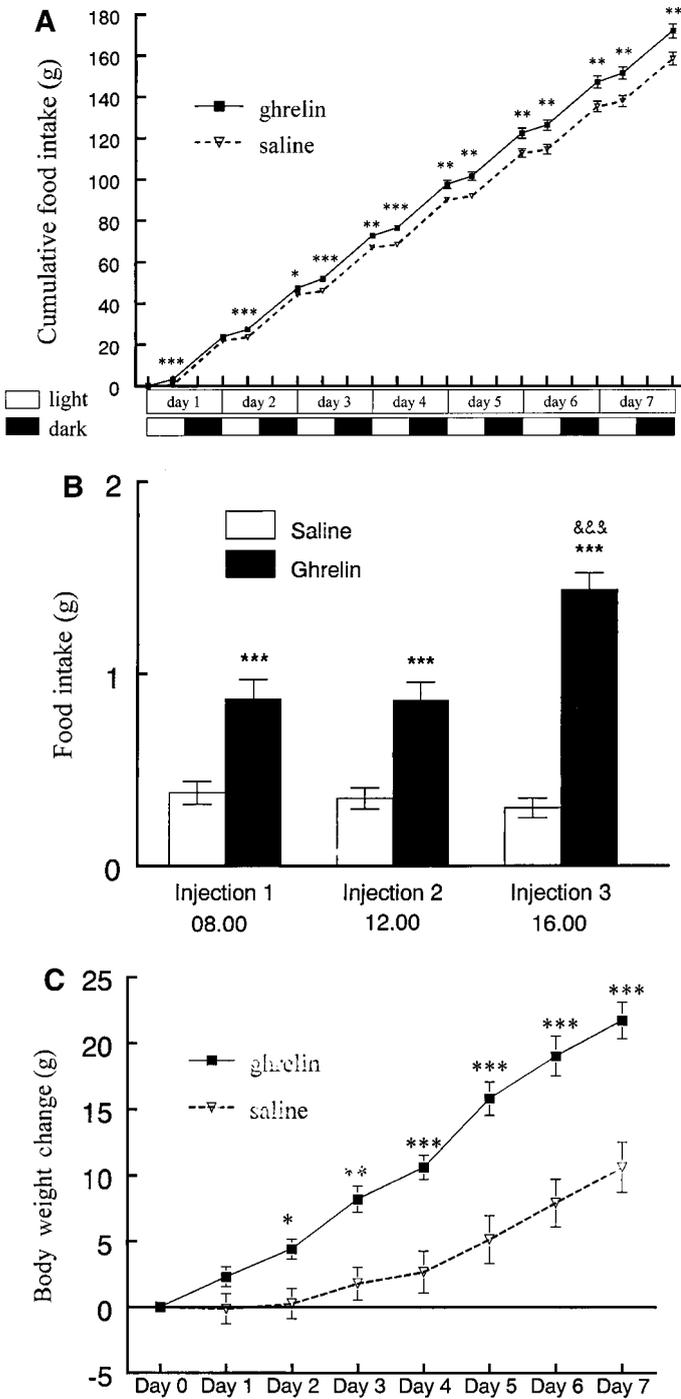


FIG. 6. Effect of intraperitoneal injection of either 10 nmol of ghrelin or saline three times daily for 7 days on cumulative food intake (A), food intake in the first hour after each timed injection (mean for all 7 days; B), and body weight change (C). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus saline; &&& $P < 0.001$ was ghrelin-treated at 08:00 h and 12:00 h.

any study day (average over 7 days: ghrelin-treated 20.7 ± 0.2 g vs. saline-treated 20.9 ± 0.2 g; NS). Ghrelin induced significant hyperphagia at 0–1 h postinjection (Fig. 6B) but not at 1–4 h between injections (ghrelin, 0.35 ± 0.05 g; saline, 0.34 ± 0.04 g). On each day of the study, the feeding response to the third injection at 1600 h was greater than that to the first two injections at 0800 h and 1200 h (Fig. 6B) (average 1-h food intake for all 7 days after each injection). The increased light-phase food intake induced by

ghrelin was associated with a marked increase in body weight gain (Fig. 6C). No adverse behavior was observed after injections.

Study 4: investigation of the effect of repeated ICV administration of ghrelin on food intake and body weight. Rats received an injection of either ghrelin or saline in the early light phase once daily for 7 days. ICV injection of 3 nmol ghrelin potently stimulated feeding during the hour after injection, with no detectable attenuation on repeated administration up to 7 days (Fig. 7A). There was a trend toward increase in 24-h food intake, which became significant by days 6 and 7 (Fig. 7B). This was associated with a significant increase in body weight in the ghrelin-treated animals by days 6 and 7 (Fig. 7C). No adverse behavior was observed.

In study 3, rats that were treated with chronic intraperitoneal ghrelin were 11.1 ± 1.4 g heavier and had eaten

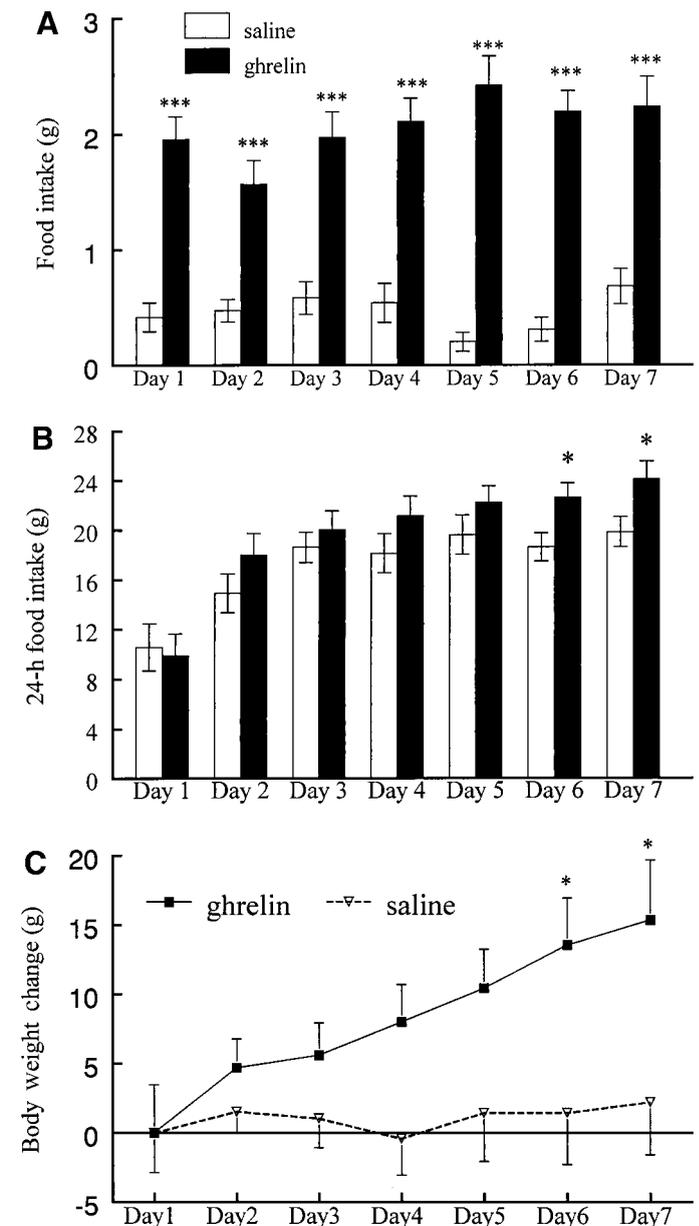


FIG. 7. Effect of once-daily ICV injection of 3 nmol of ghrelin or saline on food intake in the first hour postinjection (A), 24-h food intake (B), and body weight change (C). * $P < 0.05$, *** $P < 0.001$ versus saline.

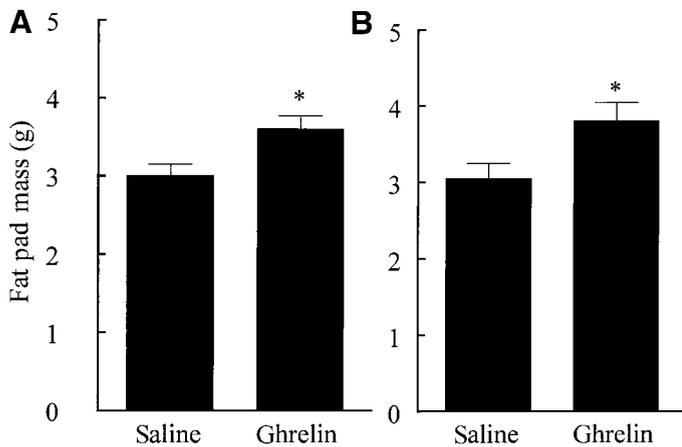


FIG. 8. Effect on testicular fat pad mass of 7 days of intraperitoneal administration of 10 nmol ghrelin or saline three times daily (A) and ICV 3 nmol ghrelin or saline once daily (B). $P < 0.05$.

13.6 ± 3.4 g more food than saline-treated rats. In study 4, chronic ICV ghrelin-treated rats were 13.1 ± 4.3 g heavier and had eaten 19.6 ± 5.5 g more than saline-treated rats. The conversion of caloric intake to body weight seems to be more efficient than in saline-treated rats, particularly for the intraperitoneal ghrelin-treated rats. The percentage caloric efficiency ([body weight gain (g)/food consumed (g)] × 100) was 12.6% for intraperitoneal ghrelin-treated rats compared with 6.7% for intraperitoneal saline-treated rats in study 3.

Study 5: investigation of the effect of repeated ghrelin administration on body composition and plasma hormones. White adipose tissue, as assessed by epididymal fat pad mass, was significantly increased after both chronic intraperitoneal ghrelin in study 3 (ghrelin-treated 3.6 ± 0.17 g vs. saline-treated 3.0 ± 0.15 g; $P < 0.05$) and after chronic ICV ghrelin in study 4 (ghrelin-treated 3.8 ± 0.24 g vs. saline-treated 3.0 ± 0.2 g; $P < 0.05$) (Fig. 8). There was no significant difference in plasma levels of GH or IGF-1 after chronic ghrelin administration, intraperitoneal (GH: ghrelin 48.7 ± 21.2 ng/ml vs. saline 42.0 ± 19.0 ng/ml, NS; IGF-1: ghrelin 1.13 ± 0.03 vs. saline 1.12 ± 0.05, NS) or ICV (GH: ghrelin 31.2 ± 10.0 ng/ml vs. saline 16.8 ± 3.0 ng/ml, NS; IGF-1: ghrelin 1.0 ± 0.07 µg/ml vs. saline 1.02 ± 0.06 µg/ml, NS). There was no evidence of organomegaly after chronic ghrelin administration as assessed by spleen and kidney weights (data not shown). Plasma TSH and total T4 were not significantly altered after chronic intraperitoneal or ICV ghrelin administration (data not shown).

DISCUSSION

In the present study, significant stimulation of food intake was observed after acute systemic administration of doses of ghrelin as low as 1 nmol per rat. There was a nonsignificant trend toward increased food intake after systemic injection of doses of ghrelin as low as 30 pmol. After systemic injection of 1 nmol ghrelin, plasma ghrelin peaked at 15 min and had returned to baseline by 2 h postinjection, paralleling the orexigenic response. The plasma concentration of ghrelin after injection of the minimum effective orexigenic dose was not significantly greater than the plasma concentration seen after a 24-h

fast. These results suggest that the stimulation of feeding by ghrelin could occur at plasma concentrations within the normal fasting range. This suggests a possible physiological role for circulating ghrelin in the control of food intake.

After systemic ghrelin administration, neuronal activation, indicated by FLI, is seen only in the Arc (11). In the current study, we showed that a very low dose (30 pmol) of ghrelin injected into the Arc potently stimulated food intake. The only other hypothalamic nucleus that showed a similarly prompt significant orexigenic response to 30 pmol ghrelin was the PVN, but here the stimulation of food intake was significantly less than that seen in the Arc. This dose of ghrelin is comparable with the lowest reported effective orexigenic dose of NPY (24 pmol) after intranuclear injection (21). Furthermore, it is much lower than the effective doses documented for many other orexigenic (18,22) and anorectic (23,24) peptides. It is worthy to note that the Arc is positioned adjacent to the median eminence, where the blood-brain barrier is deficient, and therefore can be influenced by circulating substances (14,15,25).

NPY/AgRP neurons in the medial Arc express the GHS-R (13) and exhibit FLI in response to ghrelin administration (26). In addition, recent reports demonstrated inhibition of ghrelin-induced feeding by NPY Y1-receptor antagonists (26,27) and showed upregulation of NPY (26,27) and AgRP (28) mRNA in the Arc after ghrelin administration. Thus, it seems probable that ghrelin acts, at least in part, via activation of these orexigenic neuropeptide systems. However, ghrelin still stimulates feeding in NPY knockout mice (8), and the orexigenic action of ghrelin is rapid in onset and offset in contrast to the effect of AgRP, which is relatively delayed and sustained (18). Therefore, it seems likely that ghrelin has additional actions independent of these neuronal systems. Asakawa et al. (27) recently presented data demonstrating vagal inhibition by ghrelin and inhibition of ghrelin-induced feeding and NPY expression by vagotomy in mice. However, in these studies, there was a marked reduction of food intake by vagotomy per se when comparing saline-treated vagotomized and sham-operated mice. Although vagal inhibition is an interesting possible mechanism of action for endogenous ghrelin, further investigation is required. An additional endocrine action of ghrelin cannot be excluded.

The Arc is the only identified site of ghrelin synthesis within the central nervous system (CNS) (6). After ICV administration of ghrelin, FLI is seen in the Arc, PVN, VMN, and DMN (26). This distribution corresponds to the four nuclei shown to be most sensitive to the orexigenic action of ghrelin in the present study. The observed experimental distribution of FLI may indicate a similar physiological site of action, with endogenous circulating ghrelin acting in the Arc and CNS-synthesized ghrelin acting more widely in the hypothalamus. The other nuclei that responded to 30 pmol of ghrelin, the PVN, VMN, and DMN, have all been implicated in the control of food intake (18,21,29,30). The sensitivity of the hypothalamic nuclei to the orexigenic action of ghrelin parallels expression of the GHS-R, which is particularly densely expressed in the Arc in the rat (4) and the lemur (5) but also shows clear expression in the PVN and ventromedial hypothalamus in the rat (4).

Although very small doses of ghrelin were administered into the hypothalamic nuclei, the possibility of diffusion of

peptide from one site to another must always be considered. We observed a prompt feeding response at 1 h after injection of both the Arc and the PVN. The coordinates of these nuclei are separated by a distance of 2.5 mm. Other nuclei are closer to the Arc. In particular, the AHA (at 1.9 mm from the Arc) and the DMN (at 1.2 mm from the Arc) lie between the Arc and the PVN (Fig. 1). No immediate feeding effect was seen in these nuclei, suggesting that the responses to Arc and PVN injection are distinct and not secondary to diffusion.

The DMN and VMN exhibited a delayed feeding response to 30 pmol ghrelin, observed between 1 and 2 h. However, the response was markedly less in terms of grams of food eaten than the feeding response at 1 h after Arc injection. It is theoretically possible that the delayed stimulation of feeding after administration of 30 pmol ghrelin into the VMN and DMN is due to extremely slow diffusion of peptide to the Arc. However, peptides are normally rapidly degraded in the CNS as one of the mechanisms that provide localization of neurotransmitter action. Given that in our dose finding study 30 pmol was the lowest dose that resulted in significant feeding stimulation in the Arc, diffusion is unlikely to have caused the late feeding response.

Increased food intake, body weight, and adiposity were observed after chronic administration of ghrelin either systemically or intracerebroventricularly. Hyperphagia and weight gain were recently reported after chronic ICV ghrelin administration (8,26). Chronic systemic ghrelin administration also was shown recently to cause obesity in rodents but at doses between 20 and 40 times greater than in the present study (8). This previous report failed to detect significant stimulation of food intake to account for the weight gain observed (8). This may be because food intake was measured only at 24 h. We previously reported that a single systemic injection of ghrelin increases food intake measured at 1 h postinjection but not cumulative 24-h food intake (16). Hence, a three times daily injection protocol was chosen for the current study, and this resulted in significant stimulation of 24-h food intake. Thus, our findings clarify that both ICV and systemic ghrelin cause adiposity and weight gain in part by stimulating hyperphagia.

Although acute systemic or ICV ghrelin administration causes potent stimulation of GH (16), significant stimulation of the growth axis (GH or IGF-1) was not detected after chronic administration. This is in keeping with previous data showing downregulation of the GH response to repeated GHS administration (20). As the growth axis is dynamic, we cannot exclude a subtle effect of continuous ghrelin administration. The changes in body composition, however, argue against significant sustained growth axis stimulation. Continuous ghrelin treatment did not cause organomegaly but resulted in increased fat mass, whereas adiposity is reduced after continuous GH administration (31). Others have shown that ghrelin stimulates weight gain and adiposity in GH-deficient dwarf rats (8). These data taken together suggest that ghrelin has a role in body weight homeostasis independent of stimulation of the growth axis.

Conversion of total food intake to body weight was more efficient in chronic ghrelin-treated than saline-

treated rats. This tends to suggest reduced energy expenditure in ghrelin-treated rats. Similarly, reduced energy expenditure and hence increased energy efficiency has been observed after chronic blockade or disruption of the CNS melanocortin system (32–34). Proposed underlying mechanisms include suppression of the thyroid axis, inhibition of uncoupling protein 1 expression in brown adipose tissue, and reduced locomotor activity (32,33). Others have shown that locomotor activity is not affected by continuous ICV ghrelin (26). We investigated whether suppression of the thyroid axis may account for altered energy expenditure in the current study. We previously reported that acute ICV ghrelin administration inhibits TSH at 20 min postinjection (16). However, no significant suppression of the thyroid axis was detected after chronic systemic or ICV ghrelin administration in the present study. It is not surprising to find disparate effects on the thyroid axis of acute versus continuous ghrelin administration. Rapid downregulation may occur as is seen in the growth axis after repeated GHS administration (20). However, as only a single time point was investigated, a subtle effect on the thyroid axis cannot be excluded. Previous studies (8) suggested that ghrelin administration alters respiratory quotient. Exploration of the mechanisms whereby ghrelin affects metabolism and energy expenditure is required and should be the focus of other investigations.

In conclusion, we showed that hyperphagia, weight gain, and increased adiposity occur after continuous systemic ghrelin administration at much lower doses than previously reported. This is the first report of significant feeding stimulation in response to systemic ghrelin administration, occurring at plasma ghrelin levels within the normal fasting range. The orexigenic action of ghrelin is most potent in the Arc, which is potentially accessible to the circulation. These findings are in keeping with a physiological role for circulating ghrelin in the regulation of food intake. As such, ghrelin would be an important new target for the development of treatments for obesity.

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