Ghrelin Causes Hyperphagia and Obesity in Rats.

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Abstract

Ghrelin, a circulating growth hormone releasing peptide derived from the stomach, stimulates food intake. The lowest systemically effective orexigenic dose of ghrelin is now investigated and the resulting plasma ghrelin concentration compared with that during fasting. The plasma ghrelin concentration following intraperitoneal (ip) injection of the minimum effective orexigenic dose (1nmol) was not significantly different from that occurring following a 24-hour fast. Following microinjection into defined hypothalamic sites, ghrelin (30pmol) stimulated food intake most markedly in the arcuate nucleus (0-1h 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. Following chronic systemic or intracerebroventricular (ICV) administration of ghrelin for seven days, cumulative food intake was increased (ip ghrelin 13.6 $\pm$ 3.4g greater than saline-treated, $p<0.01$; ICV ghrelin 19.6 $\pm$ 5.5g greater than saline-treated, $p<0.05$). This was associated with excess weight gain (ip ghrelin 21.7 $\pm$ 1.4g vs. saline 10.6 $\pm$ 1.9g, $p<0.001$; ICV ghrelin 15.3 $\pm$ 4.3g vs. saline 2.2 $\pm$ 3.8g, $p<0.05$) and adiposity. These data provide evidence that ghrelin is important in long-term control of food intake and body weight and that circulating fasting ghrelin stimulates food intake.

Keywords: Food Intake, Intranuclear, Arcuate nucleus, Growth, Body weight

Introduction

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 arcuate nucleus (Arc), the paraventricular nucleus (PVN) and the ventromedial nucleus (VMN) (3-5). During the development of synthetic GHSs, weight gain was noted following chronic systemic administration in immature rodents (1). Ghrelin is a circulating 28 amino acid peptide, which was recently purified from rat stomach and the gene was subsequently cloned in rats and humans (6). It is an 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 primarily synthesized in X/A-like endocrine cells in the oxyntic glands of the stomach and is present in the circulation (7). Circulating ghrelin is elevated following a 48-hour fast and subsequently lowered by administration into the stomach of 50% glucose, 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 (Arc) of colchicine treated rats (6). The arcuate nucleus is an important site in the control of food intake (9). The potent orexigenic neurotransmitters neuropeptide Y (NPY) and Agouti related protein (AgRP) are co-localized in neurons in the medial arcuate nucleus (10). Following systemic administration of ghrelin or GHSs *c-fos*-like immunoreactivty (FLI), an indicator of neuronal activation, is evident only in the arcuate nucleus (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 producing well-characterized orexigenic signals are potential targets for circulating ghrelin.

The mechanisms determining 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 arcuate nucleus (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 following acute systemic (ip) or 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 occurring during fasting, would stimulate feeding. The lowest dose of ghrelin to significantly stimulatefeeding following ip injection has been investigated and the resulting circulating ghrelin concentration compared with that seen during fasting. The arcuate nucleus 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, the effect of chronic ghrelin administration on food intake, body weight and body composition has also been examined.

Methods

Animals

Male Wistar rats (250-300g) were maintained in individual cages under controlled temperature (21-23 ^{o}C) and light (12 h light, 12h dark, lights on at 07.00h) with *ad libitum* access to food (RM1 diet, SDS UK Ltd) 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 ip injection by sham injections of saline 0.5ml on two days prior to study. For all studies rats received an injection of ghrelin or saline ip in 0.5ml volume.

Intranuclear and intracerebroventricular (ICV) cannulation and injection

Animal surgical procedures and handling were carried out as previously described (17;18). Animals were anaesthetized by ip injection of a mixture of ketamine (Ketalar HCl 60 mg/kg, Parke-Davis, Pontypool, UK) and xylazine (Rompun 12 mg/kg, Bayer UK Ltd, Bury St. Edmunds, UK) and placed in a Kopf stereotaxic frame. For intranuclear cannulation, animals were implanted with permanent 26-gauge stainless steel guide cannulae (Plastics One inc. Roanoke, VA) projecting into the Arc, PVN, VMN, medial preoptic area (MPO), supraoptic nucleus (SON), anterior hypothalamic area (AHA), dorsomedial nucleus (DMN), and lateral hypothalamic area (LHA) of the hypothalamus, according to coordinates of Paxinos and Watson (table 1, figure 1) (19). 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 μl (for intranuclear studies) or 5 μl (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 1mm below the tip of the cannulae. All substances were administered in the early light phase (09.00-10.00h). Correct intranuclear

cannula placement was confirmed histologically at the end of the study period. Following injection of 1μl black ink, animals were decapitated and brains removed and immediately frozen in liquid nitrogen and stored at –70^{o}C. Brains were sliced on a cryostat (Bright, Huntingdon, UK) into 15μM coronal sections and stained with cresyl violet. Correct ICV cannula placement was confirmed by a positive dipsogenic response to angiotensin II (150ng/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 ip dose of ghrelin that significantly stimulated feeding in freely fed rats was examined. Rats (*n* = 9-11 per group) were injected ip at 08.00 to 9.00h with saline or ghrelin (30pmol, 100pmol, 300pmol, 1nmol, 3nmol or 10nmol). Immediately after injection rats were returned to their home cages containing a pre-weighed amount of chow. The remaining food in the hopper was reweighed at 1, 2, 4 and 24 hours post-injection using an ATP Instrumentation GW 600 balance (ATP Instrumentations, Ltd., Ashby-De-la-Zouche, Leicestershire, UK) recording to the nearest 0.1g. To determine ghrelin plasma concentration, following a 72h recovery, rats (*n* = 9-11 per group) then received an ip injection of either saline or ghrelin (1nmol) at 09.00-10.00h. Animals were decapitated 15, 60 or 120min post-injection. Trunk blood was collected into plastic lithium heparin tubes containing 0.6mg aprotinin (Bayer, Haywards Heath, UK). Plasma was separated by centrifugation, frozen and stored at -70°C until radioimmunoassay (RIA). A further group of rats (n=10 per group) were either freely fed or fasted for 24 hours then killed by decapitation at 10.00-11.00h and plasma 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 (1pmol, 10pmol, 30pmol or 100pmol) at 09.00-10.00h and food intake

was measured as above. For subsequent comparison between hypothalamic nuclei a 30pmol dose was chosen as the lowest dose to significantly stimulate feeding after injection into the Arc. To examine whether nuclei failing to respond to 30pmol would respond at higher ghrelin concentrations ghrelin 300pmol was also administered. The study was of randomized cross-over design. All animals (n = 12-15/group) received saline, ghrelin 30pmol and ghrelin 300pmol by intranuclear microinjection. Animals were injected in the early light phase (09.00 to 10.00h) and food intake measured as above.

Study 3 – ***Investigation of the effect of repeated ip administration of ghrelin on food intake and body weight.***

Freely fed rats were injected ip with either saline (n = 15) or ghrelin (10 nmol, n=10) three times daily for seven days during the light phase (at 08.00h, 12.00h and 16.00h). The dose of ghrelin administered was the lowest dose to give a maximal feeding response in study 1 and in previously published data (16). Body weight was measured daily at 08.00h. Food was weighed at the time of each injection and at one hour after each injection. This allowed calculation of cumulative food intake and food intake at one hour in response to each injection. A final food and body weight was measured at 08.00h on day eight.

Study 4 – ***Investigation of the effect of repeated ICV administration of ghrelin on food intake and body weight.***

Rats were injected ICV with either saline (n = 20) or ghrelin 3nmol (n=18) once daily for seven days during the early light phase (09.00h). Ghrelin 3 nmol was chosen as the lowest dose previously demonstrated to increase 24h food intake (16). Food and body weight were determined daily just prior to injection and at 09.00h on the day following the final injection. Food was also reweighed at one hour 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 eight at 09.00-10.00h and plasma 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 NIDDK and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor University of CA, Los Angeles Medical Center) as previously described (17). Plasma ghrelin, leptin and insulin-like growth factor-1 (IGF-1) were measured using commercially available RIA kits (Phoenix Pharmaceuticals USA, Linco Research USA and Diagnostic Systems Laboratories USA respectively).

Statistics.

Results are shown as mean values ± SEM, 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 (LSD) test (Systat, Evanston, IL) was used for comparisons between three or more unpaired treatment groups, ie the feeding dose responses to ip and intra-arcuate ghrelin and the plasma ghrelin concentrations following 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 to 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 have previously shown that ip administration of ghrelin (3nmol, 10nmol or 30nmol) stimulates food intake dose-dependently. In the present study the lowest ip dose of ghrelin causing significant stimulation of food intake was 1nmol (0-2 hour food intake following ghrelin 1nmol 1.2 $\pm$ 0.2g vs. saline 0.5 $\pm$ 0.2g, $p<0.05$), although there was a non-significant trend towards increase in food intake with doses as low as 30pmol (figure 2a). In order to assess the physiological relevance of feeding stimulation by ip ghrelin we compared the plasma levels of ghrelin following ip ghrelin 1nmol with those occurring during fasting. Plasma ghrelin was significantly elevated following a 24h fast in male Wistar rats (2.79 $\pm$ 0.32 vs. fed rats 1.28 $\pm$ 0.12 pmol/ml, $p<0.001$; figure 2b). The plasma ghrelin concentration following ip injection of ghrelin 1nmol in fed rats was measured up to 2 hours (the duration of the feeding response) post-injection. The ghrelin concentrations achieved were not significantly greater than those occurring following a 24h fast (figure 2c; t=0 1.22 $\pm$ 0.13, t=15min 3.30 $\pm$ 0.23, t=60min 2.83 $\pm$ 0.12, t=120min 1.52 $\pm$ 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 30pmol (0-1h food intake 2.0 $\pm$ 0.4g vs. saline-treated 0.7 $\pm$ 0.3g, $p<0.05$, figure 3). This dose of ghrelin was, therefore, used to compare the orexigenic response between hypothalamic nuclei. To examine whether nuclei failing to respond to ghrelin 30pmol would respond at a higher ghrelin concentration a dose of 300pmol was also used. 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 following intranuclear injection was greatest at 0-1h post-injection. At 0-1h following injection of ghrelin 30pmol, food intake was significantly greater in

animals cannulated into the arcuate nucleus than into all of the other nuclei studied (figure 4; 427 ± 40% control; $p<0.001$ vs. control, $p<0.01$ vs. PVN, $p<0.001$ vs. all other nuclei). Ghrelin 30 pmol also significantly stimulated feeding at 0-1h following injection into the PVN (figure 4; 243 ± 43%, $p<0.01$), but of a lesser magnitude than observed in the Arc . The effect on food intake up to 24 hours post-injection of both doses of ghrelin into all nuclei is shown in figure 5.

The low (30pmol) 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 1-2h post-injection (figure 5). In contrast the high dose of ghrelin (300pmol) significantly stimulated feeding at 0-1h in all nuclei. This stimulation was sustained up to 2 hours in the PVN, VMN, DMN and AHA. The orexigenic response following injection of all nuclei was short-lived, with no significant stimulation of feeding observed between 2 and 24 hours post-injection.

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

Study 3 – Investigation of the effect of repeated ip administration of ghrelin on food intake and body weight.

Systemic (ip) injection of ghrelin 10nmol increased cumulative food intake when administered 3 times daily for 7 days (figure 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 any study day (average over 7 days: ghrelin-treated 20.7 $\pm$ 0.2g vs. saline-treated 20.9 $\pm$ 0.2g, p=NS). Ghrelin induced significant hyperphagia at 0-1h post-injection (figure 6b) but not at 1-4h between injections (ghrelin 0.35 $\pm$ 0.05g, saline 0.34 $\pm$ 0.04g). On each day of the study the feeding response to the third injection at 16.00h was greater than that to the first two injections at 08.00h and 12.00h (figure 6b, average one-hour food intake for all 7 days following each injection). The increased light-phase food intake induced by ghrelin was associated with a marked increase in body weight gain (figure 6c). No adverse behavior was observed following injections.

Study 4 - Investigation of the effect of repeated ICV administration of ghrelin on food intake and body weight.

Rats were injected with either ghrelin or saline in the early light phase once daily for seven days. ICV injection of ghrelin 3nmol potently stimulated feeding during the hour after injection, with no detectable attenuation on repeated administration up to seven days (figure 7a). There was a trend toward increase in 24-hour food intake, which became significant by days 6 and 7 (figure 7b). This was associated with a significant increase in body weight in the ghrelin treated animals by days 6 and 7 (figure 7c). No adverse behavior was observed.

In study 3, rats treated with chronic ip ghrelin were 11.1 $\pm$ 1.4g heavier and had eaten 13.6 $\pm$ 3.4g more food than saline treated rats. In study 4 chronic ICV ghrelin-treated rats were 13.1 $\pm$ 4.3g heavier and had eaten 19.6 $\pm$ 5.5g more than saline treated rats. The conversion of caloric intake to body weight appears to be more efficient than in saline-treated rats, particularly for the ip ghrelin-treated rats. The % caloric efficiency ((body weight gain (g)/food consumed (g)) x100) was 12.6% for ip ghrelin treated rats compared with 6.7% for ip 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 following both chronic ip ghrelin in study 3 (ghrelin-treated 3.6 $\pm$ 0.17g vs. saline-treated 3.0 $\pm$ 0.15g, $p<0.05$) and following chronic ICV ghrelin in study 4 (ghrelin-treated 3.8 $\pm$ 0.24g vs. saline-treated 3.0 $\pm$ 0.2g, $p<0.05$). There was no significant difference in plasma levels of GH or IGF1 following chronic ghrelin administration, ip (GH: ghrelin 48.7 $\pm$ 21.2ng/ml vs. saline 42.0 $\pm$ 19.0ng/ml, p=NS; IGF1: ghrelin 1.13 $\pm$ 0.03 vs. saline 1.12 $\pm$ 0.05, p=NS) or ICV (GH: ghrelin 31.2 $\pm$ 10.0 ng/ml vs. saline 16.8 $\pm$ 3.0ng/ml, p=NS; IGF1: ghrelin 1.0 $\pm$ 0.07μg/ml vs. saline 1.02 $\pm$ 0.06μg/ml, p=NS). There was no evidence of organomegaly following chronic ghrelin administration as assessed by spleen and kidney weights (data not shown). Plasma TSH was also not significantly altered following chronic ip or ICV ghrelin administration (data not shown).

Discussion

In the present study, significant stimulation of food intake was observed following acute systemic administration of doses of ghrelin as low as 1nmol per rat. There was a non-significant trend towards increased food intake following systemic injection of doses of ghrelin as low as 30 pmol. Following systemic injection of ghrelin 1nmol, plasma ghrelin peaked at 15 minutes and had returned to baseline by 2 hours post-injection, paralleling the orexigenic response. The plasma concentration of ghrelin following injection of the minimum effective orexigenic dose was not significantly greater than the plasma concentration seen following a 24-hour fast. This is the first demonstration of stimulation of feeding by ghrelin at plasma concentrations within the normal fasting range. This suggests a possible physiological role for circulating ghrelin in the control of food intake.

Following systemic ghrelin administration neuronal activation, indicated by FLI, is seen only in the arcuate nucleus (11). In the current study we show that very low dose (30pmol) ghrelin injected into the arcuate nucleus, potently stimulated food intake. The only other hypothalamic nucleus showing a similarly prompt significant orexigenic response to ghrelin 30pmol was the PVN, but here the stimulation of food intake was significantly less than that seen in the arcuate nucleus. This dose of ghrelin is comparable to the lowest reported effective orexigenic dose of NPY (24pmol) following intranuclear injection (20). Further, it is much lower than the effective doses documented for many other orexigenic (18;21) and anorectic (22;23) peptides. It is worthy of note that the arcuate nucleus is positioned adjacent to the median eminence, where the blood brain barrier is deficient, and therefore can be influenced by circulating substances (14;15;24).

NPY/AgRP neurons in the medial arcuate nucleus express the GHS-R (13) and exhibit FLI in response to ghrelin administration (25). In addition recent reports have demonstrated inhibition

of ghrelin-induced feeding by NPY Y1-receptor antagonists (25;26) and have shown up-regulation of NPY (25;26) and AgRP (27) mRNA in the Arc following 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.

The arcuate nucleus is the only identified site of ghrelin synthesis within the CNS (6). Following ICV administration of ghrelin, FLI is seen in the arcuate, PVN, VMN and DMN (25). 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 arcuate and CNS synthesized ghrelin acting more widely in the hypothalamus. The other nuclei responding to ghrelin 30pmol, the PVN, VMN and DMN, have all been implicated in the control of food intake (28;29;18;20). 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 arcuate nucleus in rat (4) and 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 one hour following injection of both the arcuate nucleus and the PVN. The co-ordinates of these nuclei are separated by a distance of 2.5mm. Other nuclei are closer to the Arc. In particular the AHA (at 1.9mm from the Arc) and the DMN (at 1.2 mm from the Arc) lie between the Arc and the PVN (figure 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 ghrelin 30pmol, observed between 1 and 2 hours. However, the response was markedly less in terms of grams of food eaten than the feeding response at 1 hour following Arc injection. It is theoretically possible that the delayed stimulation of feeding following administration of ghrelin 30pmol 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 providing localization of neurotransmitter action. Given that in our dose finding study 30pmol was the lowest dose resulting in significant feeding stimulation in the arcuate nucleus, diffusion is unlikely to have caused the late feeding response.

Increased food intake, body weight and adiposity were observed following chronic administration of ghrelin either systemically or ICV. Hyperphagia and weight gain have recently been reported following chronic ICV ghrelin administration (8;25). Chronic systemic ghrelin administration has also recently been shown 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 only measured at 24 hours. We have previously reported that a single systemic injection of ghrelin increases food intake measured at 1 hour post-injection but not cumulative 24-hour food intake (16). Hence, a three times daily injection protocol was chosen for the current study and this resulted in significant stimulation of 24-hour 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 following chronic administration. This is in keeping with previous data showing down-regulation of the GH response to repeated GHS administration (30). The change in body composition of rats following chronic ghrelin administration, however, argues against significant sustained growth axis stimulation. Chronic ghrelin treatment did not cause organomegaly but resulted in increased fat mass whereas adiposity is reduced following chronic GH administration (31). Others have shown that ghrelin stimulates weight gain and adiposity in GH-deficient dwarf rats (8). Thus our data, taken together with previous publications, suggest that the role of ghrelin in body weight homeostasis is 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 would tend to suggest reduced energy expenditure in ghrelin-treated rats. Others have shown that locomotor activity is not affected by chronic ICV ghrelin (25). We investigated whether suppression of the thyroid axis may account for altered energy expenditure. It has previously been reported that acute ICV ghrelin administration inhibits TSH at 20 minutes post-injection (16). However no significant suppression of the thyroid axis was detected following chronic systemic or ICV ghrelin administration in the present study.

In conclusion we have shown that hyperphagia, weight gain and increased adiposity occur following chronic 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 arcuate nucleus, 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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Legends

Table 1. Coordinates (mm) of the hypothalamic areas cannulated for intranuclear injection, as taken from rat brain atlas. (19)

Figure 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*).

Figure 4. (a) Food intake 0-2 hours after ip injection of saline or ghrelin 0.03 to 10nmol), * $p<0.05$, *** $p<0.001$ vs. saline control. Comparison of plasma ghrelin concentration following (b) 24h fast or (c) ip injection of ghrelin 1nmol in freely fed rats, *** $p<0.001$ vs. freely fed and uninjected controls respectively.

Figure 3 Food intake at one hour post-injection of saline or ghrelin (1 to 100pmol) into the arcuate nucleus. * $p<0.05$, ** $p<0.01$ vs. saline.

Figure 4. Comparison between hypothalamic nuclei of food intake stimulated by ghrelin 30pmol at 0-1h post-injection. Food intake expressed as % of control. Control food intake indicated by dotted line. •• $p < 0.01$ vs. PVN, $p<0.001$ vs all other nuclei; *** $p<0.001$ vs saline, ** $p<0.01$ vs saline.

Figure 5. Effect on food intake of injection of ghrelin 30pmol (hatched bars) or 300pmol (filled bars) into a)Arc, b)PVN, c)LHA, d)MPO, e) DMN, f) AHA, g) SON, h) VMN over a 24h period in freely fed rats. Food intake expressed as % saline control with control food intake (100%) indicated by dotted line. ** $p<0.01$, *** $p<0.001$ vs. saline.

Figure 6 Effect of ip injection of either ghrelin 10nmol or saline three times daily for seven days on (a) cumulative food intake, (b) food intake in the first hour after each timed injection (mean for all seven days), (c) body weight change * p <0.05, **p<0.01, ***p<0.001 vs. saline; $^{\&\&\&}$p<0.001 was ghrelin-treated at 08.00h and 12.00h.

Figure 7. Effect of once daily ICV injection of ghrelin 3nmol or saline on: (a) food intake in the first hour post-injection, (b) 24h food intake (c) body weight change. *<0.05, **p<0.01, ***p<0.001 vs. saline.

TARGETED AREA	POSTERIOR FROM BREGMA	LATERAL FROM MIDLINE	DEPTH
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**