Peripheral Oxyntomodulin Reduces Food Intake and Body Weight Gain in Rats

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Oxyntomodulin (OXM) is a circulating gut hormone released post prandially from cells of the gastrointestinal mucosa. Given intracerebroventricularly to rats, it inhibits food intake and promotes weight loss. Here we report that peripheral (ip) administration of OXM dose-dependently inhibited both fast-induced and dark-phase food intake without delaying gastric emptying. Peripheral OXM administration also inhibited fasting plasma ghrelin. In addition, there was a significant increase in c-*fos* immunoreactivity, a marker of neuronal activation, in the arcuate nucleus (ARC). OXM injected directly into the ARC caused a potent and sustained reduction in refeeding after a fast. The anorectic actions of ip OXM were blocked by prior intra-ARC administration of the glucagon-like peptide-1 (GLP-1) receptor antagonist, exendin$_{9–39}$, suggesting that the ARC, lacking a complete blood-brain barrier, could be a potential site of action for circulating OXM. The actions of ip GLP-1, however, were not blocked by prior intra-ARC administration of exendin$_{9–39}$, indicating the potential existence of different OXM and GLP-1 pathways. Seven-day ip administration of OXM caused a reduction in the rate of body weight gain and adiposity. Circulating OXM may have a role in the regulation of food intake and body weight. (*Endocrinology* 145: 2687–2695, 2004)

O XYNTOMODULIN (OXM) IS a product of proglucagon processing in the intestine and central nervous system (CNS), released from intestinal L-cells with the putative satiety factor, glucagon-like peptide-1 (GLP-1) (1). It is also produced in the neurons of the nucleus of the solitary tract (NTS) of the brain stem (2). The structure of OXM comprises the entire glucagon sequence with a C-terminal basic octapeptide extension named spacer peptide-1 (also known as KA-8). OXM, originally isolated from porcine gut (3, 4), was found to be a potent inhibitor of pentagastrin-stimulated gastric acid secretion and gastric emptying in rodents and man (5–7). Despite the presence of OXM-like-immunoreactivity in CNS, notably in the hypothalamus (8), little is known about the physiological role of OXM. We have shown that intracerebroventricular and intranuclear administration of low doses of OXM caused a robust and sustained inhibition of food intake in fasted rats (9). Recently, we reported that repeated daily intracerebroventricular administration of OXM to rats caused a marked reduction in body weight gain and body adiposity, compared with pair-fed rats (10), suggesting that OXM is a potential regulator of appetite and body weight.

Another proglucagon-derived gut hormone, GLP-1, is an inhibitor of food intake in rats when injected intracerebroventricularly (11, 12). However, these anorectic actions are not only observed after administration directly into the CNS; systemic administration (iv and sc) of GLP-1 to rodents and humans (13–15) also leads to a reduction in food intake. Similarly, we recently reported that PYY$_{3–36}$, a gut hormone in the pancreatic polypeptide/neuropeptide Y (NPY) family, inhibits food intake when given peripherally (16).

It has been suggested that some circulating gut-derived peptides can alter the activity of neurons within the arcuate nucleus (ARC) of the hypothalamus (17). The blood-brain barrier is incomplete in this area, and the ARC is therefore able to sense changes in nutritional status and circulating factors (18). After peripheral administration of the orexigenic gut hormone ghrelin, expression of the early immediate gene, c-*fos* (a marker of neuronal activation) was increased and observed almost exclusively in the ARC NPY-containing neurons (19, 20). Circulating PYY$_{3–36}$ also appears to alter the activity of the NPY and proopiomelanocortin (POMC) neurons in the ARC (16). Thus, circulating gut hormones may alter the central appetite circuits found within the ARC.

OXM is released postprandially with the gut peptides GLP-1 and PYY$_{3–36}$, which are reported to inhibit food intake and decrease body weight after peripheral administration (14–16). To examine whether OXM had similar actions, we administered OXM peripherally to rodents and investigated both its acute and chronic effect on food intake and body weight. It has been reported that PYY$_{3–36}$ and ghrelin alter the activity of the hypothalamic appetite neurons found in the ARC (16, 20), whereas GLP-1 has been reported to act via brain stem pathways and decrease gastric motility (2). Thus, we investigated some of the potential mechanisms of OXM action by measuring c-*fos*-like immunoreactivity in the brain after peripheral administration, neuropeptide release from *ex vivo* hypothalamic explants, gastric emptying, and the effect

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Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; BAT, brown adipose tissue; CCK, cholecystokinin; CNS, central nervous system; FLI, fos-like IR; GLP-1, glucagon-like peptide-1; IR, immunoreactivity; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; OXM, oxyntomodulin; POMC, proopiomelanocortin; WAT, white adipose tissue.

of OXM on food intake after direct administration into the ARC.

Materials and Methods

Peptides and chemicals

Human OXM (molecular mass = 4420) was purchased from Bachem UK Ltd. (Meyerside, UK). GLP-1 (molecular mass = 3297) was purchased from Peninsula Laboratories Inc. (St. Helens, UK). Exendin$_{9-39}$ was synthesized by Dr. P. Byfield (Medical Research Council, Hemostasis Unit, Clinical Sciences Center, Hammersmith Hospital, London, UK) using F-moc chemistry on a 396 MPS peptide synthesizer (Advanced ChemTech Inc., Louisville, KY) and purified by reverse-phase HPLC on a C_8 column (Phenomex, Macclesfield, UK), using a gradient of acetonitrile on 0.1% trifluoroacetic acid. Correct molecular weight was confirmed by mass spectrometry. Chemicals were purchased from VWR International Ltd. (Leicestershire, UK) unless otherwise stated.

Animals

Adult male Wistar rats were maintained in individual cages under controlled conditions of temperature (21–23 C) and light (lights on 0700 h; lights off 1900 h) with *ad libitum* access to standard rat chow (RM1 diet, Special Diet Services UK Ltd., Witham, Essex, UK) and water unless stated otherwise. All procedures were approved by the British Home Office Animals (Scientific Procedures) Act 1986 (Project Licenses PPL: 90/1077 and 70/5281) and the Ethical Review Committee. At all times the minimum number of animals was used.

Intra-ARC cannulation

Rats (350–400 g) had permanent indwelling, unilateral, stainless steel guide cannulae (Plastics One, Roanoke, VA) stereotactically implanted into the ARC of the hypothalamus, using a protocol described previously (21) (coordinates relative to bregma: 0.3 mm lateral, 3.3 mm posterior, and 9.0 mm below the outer surface of the skull). Cannula placement was determined (by injection of ink) and test substances were administered as previously described [1 μl solution/min delivered (total volume = 1 μl) via SILASTIC brand silicon tubing (Dow Corning Corp., Midland, MI) attached to a peristaltic pump (21)]. Data from animals whose cannula were found to be incorrectly placed were excluded from analyses (>75% correct cannula placement).

Intraperitoneal injections

Rats (180–200 g) received ip injections using a 1-ml syringe and a 25-gauge needle. The maximum volume of injection was 500 μl and was adjusted according to the weight of the individual animal. All peptides were dissolved in saline.

In vivo protocols

Experiment 1: investigating the dose-response effect of peripheral administration of OXM on food intake in fasted rats. Rats were fasted for 24 h before study. During the early light phase (0900–1000 h), rats were given a single ip injection of saline or OXM (3, 10, 30, or 100 nmol/kg body weight) (n = 12/group). After injection, the rats were returned to their home cages and provided with a preweighed amount of chow. Food intake was measured 1, 2, 4, 8, and 24 h post injection.

Experiment 2: investigating the effect of peripheral administration of OXM on food intake in nonfasted rats during the dark phase. The dark phase is the normal feeding time for rats. Any inhibition of food intake at this time could be considered to be more physiological than alterations to refeeding after a fast. Rats received a single ip injection of saline or OXM (3, 10, 30, or 100 nmol/kg body weight) (n = 12/group) before lights out (1800–1900 h). Food intake was measured 1, 2, 4, 8, and 12 h post lights-out.

Experiment 3: comparative dose-response curves for ip OXM and GLP-1. The effects of ip OXM and GLP-1 on food intake were compared. Rats were fasted for 24 h before the study. During the early light phase (0900–1000 h), rats (n = 12–14/group) received an ip injection of 1) saline or OXM

(3, 10, 30, or 100 nmol/kg) or, on a separate occasion, 2) saline or GLP-1 (3, 10, 30, or 100 nmol/kg). On both occasions, food intake was measured 1 h post injection.

Experiment 4: the effect of repeated ip injections of OXM. Rats were randomized by weight into three groups (n = 15/group): 1) Saline treated (ip saline) with *ad libitum* access to food; 2) OXM treated (ip OXM, 50 nmol/kg body weight per injection, an effective but submaximal dose based on pilot experiments) with *ad libitum* access to food; and 3) Pair fed (saline treated but food restricted to the mean light- and dark-phase food intake of the OXM-treated group). Rats were injected twice daily (0700 and 1800 h) for 7 d. Food intake (grams), body weight (grams), and water intake (milliliters) were measured daily. On the eighth day, the rats were killed by decapitation. Epididymal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) were removed and weighed as an assessment of body adiposity.

Experiment 5: investigating the effect of peripheral administration of OXM on gastric emptying. The effect of OXM on gastric emptying was investigated using a protocol derived from Barrachina *et al.* (22). Rats were fasted for 36 h to ensure that the stomach was empty. During the early light phase (0900–1000 h), they were allowed *ad libitum* access to a preweighed amount of standard rat chow for 30 min. After that time, the food was removed and reweighed. The rats then received an ip injection of saline, OXM (50 nmol/kg body weight, a submaximal but effective dose), GLP-1 (50 nmol/kg body weight), or cholecystokinin (CCK)-8 (15 nmol/kg body weight, a dose known to be effective in a previous in-house experiment). Rats were killed by CO_2 at the same times as those used in the previous feeding studies: 1, 2, 4, or 8 h post feeding (n = 12/group per time point). The CCK-8 group was used as a positive control for the experiment at the 2-h time point only and GLP-1 at the 1-h time point. A laparotomy was rapidly performed and the stomach exposed. The pyloric junction was ligated (2.0 Mersilk, Johnson & Johnson, Brussels, Belgium), followed by ligation of the gastroesophageal junction, and the stomach was removed. The gastric contents were removed, placed in a preweighed weighing boat, and left to air dry for 48 h. When dry, the contents were weighed and the percentage of the chow ingested during the 30-min refeeding period remaining in the stomach per rat was then calculated using the following formula:

Percentage food remaining in the stomach

$$= \frac{\text{dry weight of stomach content}}{\text{weight of food ingested}} \times 100$$

Experiment 6: investigating the effect of increasing doses of intra-ARC OXM. Intra-ARC cannulated rats were randomized by weight into six groups (n = 12–15/group). During the early light phase (0900–1000 h), 24-h fasted rats received an intra-ARC injection of saline or OXM (0.01, 0.03, 0.1, 0.3, or 1.0 nmol). Food intake was measured 1, 2, 4, 8, and 24 h post injection.

Experiment 7: investigating whether peripherally administered OXM is acting directly via ARC GLP-1 receptors. Intra-ARC cannulated rats were randomized into six groups (n = 10–12/group). During the early light phase (0900–1000 h), 24-h fasted rats received an intra-ARC injection of saline or exendin$_{9-39}$ [5 nmol in 1 μl saline, a dose shown previously to block the effects of GLP-1 when coadministered into a hypothalamic nucleus (9)] followed by an ip injection of saline, OXM (30 nmol/kg body weight), or GLP-1 (30 nmol/kg body weight) 15 min later.

Experiment 8: investigating the plasma OXM-immunoreactivity (IR) and ghrelin-IR levels after ip administration of OXM. OXM or saline was administered to fasted rats to investigate the plasma OXM-IR and ghrelin-IR levels after ip OXM. Plasma OXM-IR levels were measured, using a previously described assay, which also measures enteroglucagon (*i.e.* N-terminally elongated OXM) (23). The OXM-IR assay (23) could detect changes of 10 pmol/liter (95% confidence limit) with an intraassay variation of 5.7%. The ghrelin RIA (24) measured both octanoyl and des-octanoyl ghrelin (total ghrelin). It did not cross-react with any known gastrointestinal or pancreatic peptide hormones and could detect changes of 10 pmol/liter (95% confidence limit) with an intraassay variation of 9.5%.

Rats (n = 10/group) were ip injected with OXM (30 nmol/kg and 100

nmol/kg) or saline at the beginning of the light phase. The rats were decapitated 30 or 90 min after the ip injection and trunk blood collected. All plasma was collected and frozen at −20 C until assayed for OXM-IR and ghrelin-IR. During the entire postinjection period, the rats remained fasted. The time points and doses of OXM were chosen by reference to previous feeding studies (see experiment 1).

The release of gut hormones has been found to be influenced by the content of the diet, in particular the fat content. For this reason, a further three groups (n = 10) were investigated: 1) rats fasted overnight and killed at the beginning of the light phase, 2) rats fed high-fat rat chow (45% fat, Research Diets Inc., New Brunswick, NJ) overnight and decapitated at the beginning of the light phase, 3) rats fasted overnight and at lights-on given *ad libitum* access to 45% high-fat chow for 2 h. The rats were decapitated at the end of this 2-h high-fat meal (*i.e.* 2 h into the light phase). All plasma was collected and frozen at −20 C until assayed for OXM-IR and ghrelin-IR.

Immunohistochemical measurement of fos-like IR

Full details of the protocol for the measurement of fos-like IR (FLI), as a marker of neuronal activation, is described elsewhere (25). Briefly, 90 min after an ip injection of OXM (50 nmol/kg), CCK (15 nmol/kg), or saline (n = 8/group), rats were terminally anesthetized and transcardially perfused with PBS [0.1 M (pH 7.4)] followed by 4% paraformaldehyde in PBS. The brains were removed and postfixed overnight in 4% paraformaldehyde in PBS and then transferred to sucrose in PBS (20% wt/vol) overnight. Serial frozen 40-μm coronal sections of brain and brain stem were cut on a freezing microtome and stained for FLI with *c-fos* Ab-5 (1:100,000; Oncogene, Santa Cruz, CA; incubated overnight at 4 C). After incubation with primary antibody, sections were incubated for 1 h at room temperature with biotinylated rabbit antirabbit IgG (1:600: Vector Laboratories, Peterborough, UK) followed by a further 1 h with avidin-biotin-peroxidase complex (4.5 μl of both reagent A and B per milliliter of diluent; Vector Laboratories). After washing, sections were immersed in Chromagen solution containing nickel sulfate intensified diaminobenzidine tetrahydrochloride and hydrogen peroxide for approximately 10 min. The sections were then mounted on poly-L-lysine-coated slides, dehydrated in increasing concentrations of ethanol (50–100%), delipidated in xylene, and coverslipped using DPX mountant. Slides were examined for FLI-positive nuclei using a light microscope and images captured using a microimager. The number of FLI-positive nuclei in the hypothalamus and brain stem were counted by an investigator blind to the experimental groups. The location of each nucleus was defined by comparison with a previous text (25a).

Hypothalamic explant static incubation

To investigate the effect of OXM on the release of hypothalamic peptides involved in the regulation of feeding, hypothalami were removed, incubated with OXM, and the subsequent release of peptide measured. The static incubation system used is described elsewhere (26). Briefly, male Wistar rats (n = 60) were killed by decapitation and the whole brain removed. The brain was mounted, ventral surface uppermost, on a vibrating microtome (Microfield Scientific Ltd., Dartmouth, UK). A 1.7-mm slice was taken from the basal hypothalamus, blocked lateral to the Circle of Willis, and incubated in chambers containing 1 ml of artificial cerebrospinal fluid (aCSF), which was equilibrated with 95% O_2 and 5% CO_2. The hypothalamic slice encompassed the medial preoptic area, paraventricular nucleus, dorsomedial nucleus, ventromedial nucleus, lateral hypothalamus, and ARC. The tubes were placed on a platform in a water bath maintained at 37 C. After a 2-h equilibration period, each explant was incubated for 45 min in 600 μl aCSF (basal period) before being challenged with OXM, 100 nM [a dose representing approximately 10 times the IC_{50} of OXM for the GLP-1 receptor (9)]. The viability of the tissue was confirmed by a final 45-min exposure to aCSF containing 56 mM KCl. At the end of each period, the aCSF was removed and stored at −20 C until assayed for αMSH-IR and NPY-IR as indicators of the activity of the appetite-regulatory neurons in the ARC. αMSH is the main product of the appetite inhibitory POMC neurons, and NPY is a product of the appetite stimulatory NPY neurons.

RIA to measure αMSH-IR and NPY-IR

αMSH-IR was measured using an in-house RIA, developed using an antibody from Chemicon International Inc. (Temecula, CA) (27). NPY-IR was measured using an in-house RIA and antibody described previously (28).

Statistical analyses

Data from ip and intra-ARC feeding studies were analyzed by one-way ANOVA with *post hoc* least significant difference test using SYSTAT (version 10; SYSTAT Software, Inc., London, UK). Fat pad weights (expressed as a percentage of body weight) from different treatment groups were analyzed using an unpaired Student's *t* test. Data from the hypothalamic explant incubation studies, in which each explant was compared with its own basal period, were analyzed by paired Student's *t* test. In all cases, $P < 0.05$ was considered to be statistically significant.

Results

Effect of peripheral administration of OXM in fasted rats

Intraperitoneal administration of OXM (30 nmol/kg and 100 nmol/kg) caused a significant inhibition in refeeding in 24-h fasted rats 1 h post injection, compared with saline controls [1 h: OXM 30 nmol/kg, 5.4 ± 0.2 g ($P < 0.05$), 100 nmol/kg, 4.5 ± 0.2 g ($P < 0.05$) *vs.* saline, 6.3 ± 0.2 g]. The reduction in food intake caused by 30 nmol/kg was sustained until 8 h post injection. However, the highest dose of OXM (100 nmol/kg) continued to significantly inhibit food intake 24 h post injection (24 h: OXM, 100 nmol/kg, 33.3 ± 0.9 g *vs.* saline, 40.4 ± 2.0 g; $P < 0.05$) (Fig. 1A). The 3 nmol/kg and 10 nmol/kg failed to alter food intake at any time point investigated. No adverse behaviors were noted after OXM treatment in accordance with our previously published findings (9).

Effect of peripheral administration of OXM in nonfasted rats on dark phase food intake

OXM, 3 and 10 nmol/kg, failed to affect food intake at any time point investigated in nocturnally feeding rats injected immediately before the dark phase (data not shown). However, OXM, 30 nmol/kg, significantly inhibited food intake until 2 h post injection (2 h: OXM, 30 nmol/kg, 4.5 ± 0.4 g *vs.* saline, 5.8 ± 0.4 g; $P < 0.05$). Food intake was reduced 4 h post injection, but this was not significant. OXM, 100 nmol/kg, significantly inhibited food intake throughout the dark phase (12 h: OXM, 100 nmol/kg, 22.3 ± 0.6 g *vs.* saline, 25.6 ± 0.7 g; $P < 0.05$) (Fig. 1B).

Effect of repeated ip administration of OXM

Twice-daily ip injections of OXM (50 nmol/kg) for 7 d caused a decrease in cumulative daily food intake, compared with saline-treated control rats (d 7: OXM, 168 ± 4.6 g *vs.* saline, 180 ± 4.3 g; $P < 0.01$) (Fig. 2A). OXM-treated rats gained weight significantly more slowly than saline controls (d 7: OXM, 21.0 ± 1.5 g *vs.* saline, 37.6 ± 1.9 g; $P < 0.005$). The pair-fed rats gained more weight than the OXM-treated rats, despite receiving the same food intake [d 7: pair-fed, 33.5 ± 2.0 g; $P = $ NS *vs.* saline (*ad libitum* fed), $P < 0.05$ *vs.* OXM] (Fig. 2B). In addition, chronic OXM caused a reduction in adiposity, compared with pair-fed rats [WAT: OXM, 0.51 ± 0.01 g (percent body weight) *vs.* pair fed, 0.61 ± 0.02 g; $P < 0.05$; BAT: OXM, 0.12 ± 0.01 g *vs.* pair fed, 0.15 ± 0.01; $P < 0.05$], suggesting that OXM caused additional metabolic
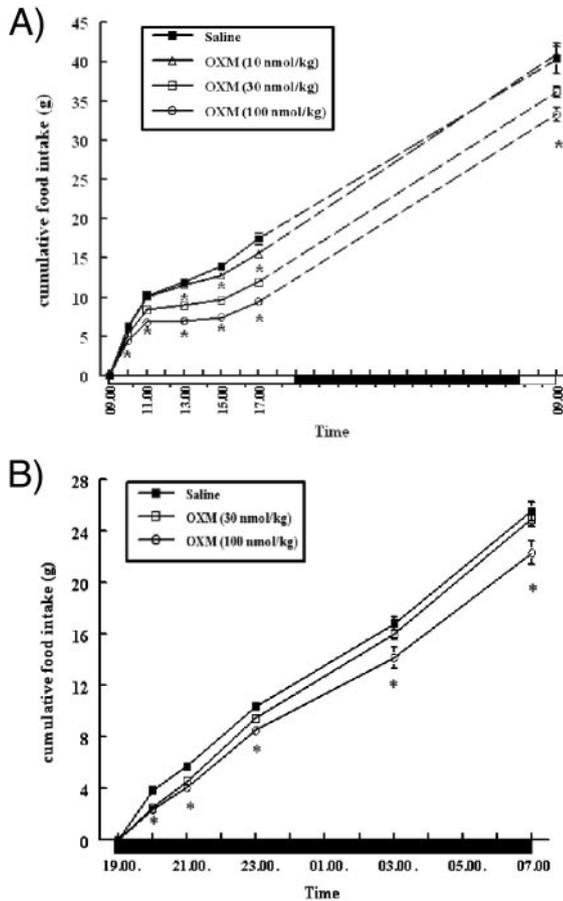
FIG. 1. A, The effect of ip OXM or saline on cumulative food intake (grams) in 24-h fasted rats injected during the early light phase (n = 12/group. Bar along x-axis, *open bar*, light phase (lights-on), *closed bar*, dark phase (lights-off). *Dashed lines* between 1700 and 0900 h represent the passing of time, but food intake was not measured during this period. B, ip OXM or saline on cumulative food intake in nonfasted rats injected before the onset of the dark phase (n = 12/group). *Closed bar along x-axis*, dark phase. *, P < 0.05 *vs.* saline.
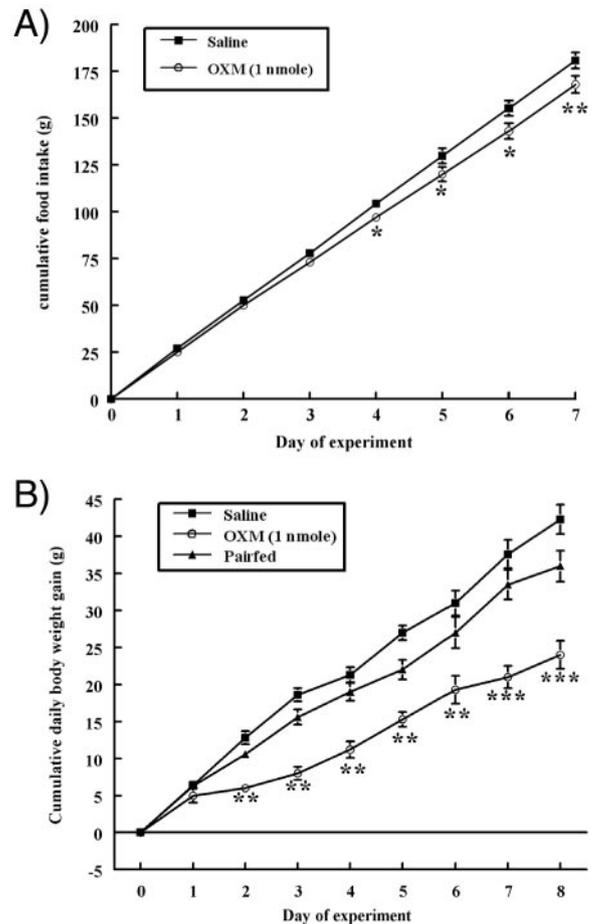

FIG. 2. The effect of twice daily ip injections of OXM (50 nmol/kg) or saline for 7 d on cumulative food intake (A) and body weight gain (grams) (B) (n = 15 per group). *, P < 0.05; **, P < 0.01; ***, P < 0.005 *vs.* saline.

effects. Water intake was reduced in OXM-treated rats on d 1 and 2 (d 1: OXM, 24.1 ± 1.3 ml *vs.* saline, 28.1 ± 1.3 ml; P < 0.05). On subsequent days, there was an increase in daily water intake, compared with saline-treated rats (d 3–6). However, by d 7, there was no difference in cumulative water intake between saline and OXM-treated groups (d 7: OXM, 192 ± 1.1 ml *vs.* saline, 190 ± 2.0 ml; P = NS).

Comparative dose responses for ip OXM and GLP-1

Intraperitoneal administration of OXM, 30 and 100 nm/kg, caused a significant reduction in food intake (P < 0.05 and P < 0.01, respectively). Ten nanomoles per kilogram of OXM tended to reduce food intake, although this was not statistically significant (Fig. 3A). Intraperitoneal administration of GLP-1 also reduced fast-induced food intake, although this was significant only at a dose of 100 nmol/kg (P < 0.05) (Fig. 3B).

Role of delayed gastric emptying on the anorectic effect of OXM

One hour after food was presented to the 36-h fasted rats, the dry weight of the contents of the stomachs (as a percent-

age of the food consumed during the 30-min feeding period) of GLP-1-treated rats were significantly greater than that of saline-treated rats (1 h: GLP-1, 76.9 ± 2.7% *vs.* saline, 65.8 ± 1.6%; P < 0.01), suggesting that GLP-1 caused a significant decrease in gastric emptying. The stomach contents of OXM-treated rats were greater than those of saline-treated controls, although this was not statistically significant (1 h: OXM, 72.0 ± 1.4% *vs.* saline 65.8 ± 1.6%; P = 0.07), suggesting that OXM has a slight effect on gastric emptying. Two hours post feeding, OXM did not affect the emptying of the contents of the stomach, compared with saline-treated rats. However, rats injected with the positive control for this time point, CCK (15 nmol/kg) had significantly greater stomach content (2 h: CCK, 64.7 ± 6.4% *vs.* saline, 38.5 ± 5.3%; P < 0.01), confirming that CCK caused a significant decrease in the rate of gastric emptying in this model. There was no effect of OXM on the emptying of the stomach contents, compared with saline-treated rats, at 4 or 8 h post feeding (data not shown).

Investigating the effect of OXM injected intra-ARC

Food intake was significantly inhibited by all doses (except 0.01 nmol) of intra-ARC OXM during the first hour of refeeding after a 24-h fast [1 h: OXM 0.03 nmol, 6.1 ± 0.5 g (P < 0.05);
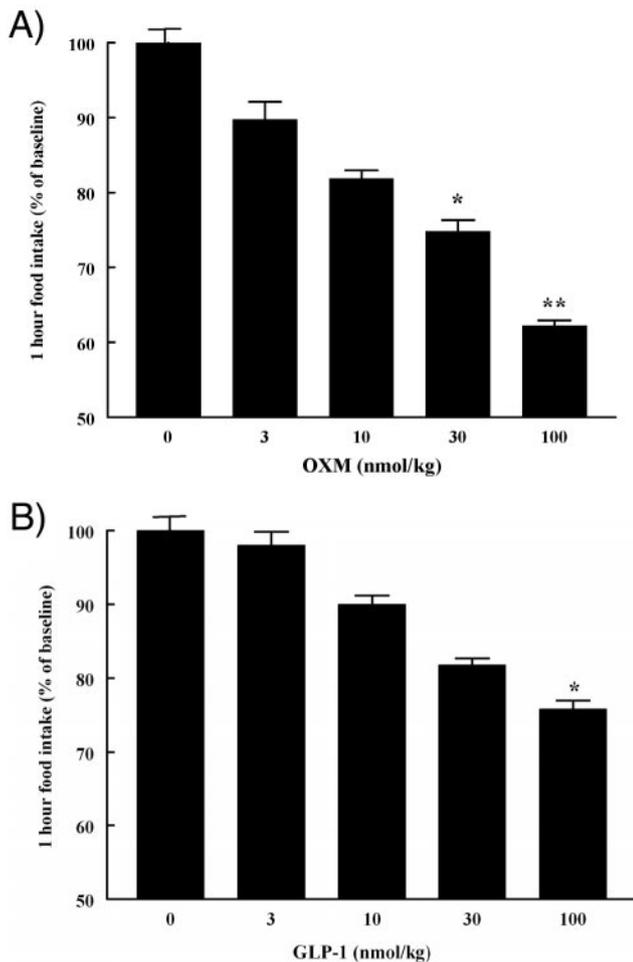
FIG. 3. Comparative dose-response curves for ip OXM and GLP-1 (0, 3, 10, 30, and 100 nmol/kg) in fasted rats during the early light phase, expressed as a percentage of baseline food intake (n = 12–14/group). *, $P < 0.05$; **, $P < 0.01$ *vs.* control group (0 nmol/kg).
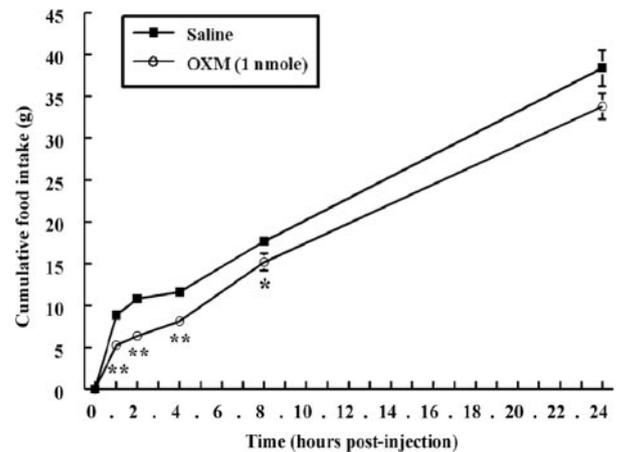

FIG. 4. The effect of OXM (1.0 nmol) or saline on cumulative 24-h food intake when administered into the ARC of 24-h fasted rats (n = 12–15/group). *, $P < 0.05$; **, $P < 0.01$ *vs.* saline.
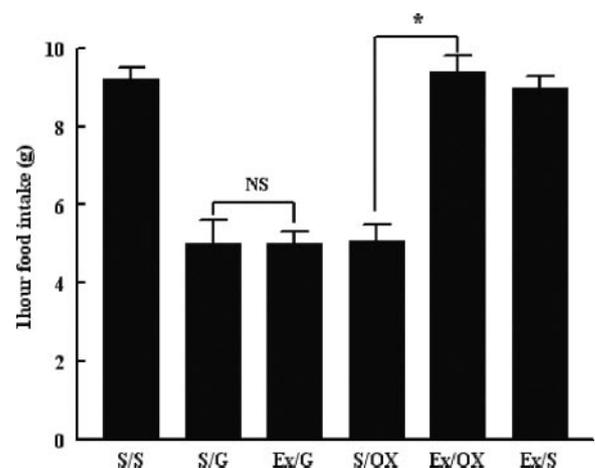

FIG. 5. The effect of intra-ARC administration of exendin$_{9-39}$ (5 nmol) or saline injected 15 min before ip administration of OXM (30 nmol/kg), GLP-1 (30 nmol/kg), or saline on 1-h food intake (grams) (n = 10–12/group). S, Saline; G, GLP-1 (30 nmol/kg); OX, OXM (30 nmol/kg); Ex, exendin$_{9-39}$ (5 nmol). *, $P < 0.01$ saline/OXM *vs.* exendin$_{9-39}$/OXM.

0.1 nmol, 5.6 ± 0.4 g ($P < 0.05$); 0.3 nmol, 5.1 ± 0.6 g ($P < 0.01$); 1.0 nmole, 3.6 ± 0.5 g ($P < 0.005$) all *vs.* saline, 7.7 ± 0.2 g] (Fig. 4). OXM 0.3 and 1.0 nmol continued to inhibit food intake until 8 h post injection. Twenty-four hours post injection, food intake was still lower after OXM 1.0 nmol, although this was not significant (24 h: OXM, 1.0 nmol, 37.8 ± 3.0 g *vs.* saline, 40.8 ± 1.6 g; P = NS).

Investigating whether peripherally administered OXM is acting via ARC GLP-1 receptors

Intraperitoneal administration of both GLP-1 and OXM (30 nmol/kg) caused a reduction in refeeding 1 h post injection [1 h: GLP-1, 5.0 ± 0.6 g ($P < 0.01$); OXM, 5.1 ± 0.4 g ($P < 0.01$) *vs.* saline, 9.2 ± 0.3 g]. However, the anorexia caused by ip administration of OXM was blocked by prior administration of the GLP-1 receptor antagonist, exendin$_{9-39}$, injected directly into the ARC (OXM alone, 5.1 ± 0.4 g *vs.* OXM + exendin$_{9-39}$, 9.4 ± 0.4 g; $P < 0.01$) (Fig. 5). Inhibition of food intake by ip GLP-1 was not affected by prior intra-ARC administration of exendin$_{9-39}$ (GLP-1 alone, 5.0 ± 0.6 g *vs.* GLP-1 + exendin$_{9-39}$, 5.0 ± 0.3 g; P = NS).

Investigating plasma OXM-IR and ghrelin-IR levels after ip administration of OXM

Intraperitoneal administration of OXM (30 and 100 nmol/kg) increased plasma OXM-IR 30 and 90 min post injection [30 min plasma OXM-IR pmol/liter: saline 61.8 ± 8.9, OXM 30 nmol/kg 448.9 ± 184.4, OXM 100 nmol/kg 997.1 ± 235.4; 90-min plasma OXM-IR pmol/liter: saline 47.5 ± 4.5, OXM 30 nmol/kg 150.6 ± 52.5, OXM 100 nmol/kg 107.8 ± 25.0] (Fig. 6).

The plasma OXM-IR levels were determined in three additional groups: 1) rats fasted overnight and killed at the beginning of the light phase (plasma OXM-IR pmol/liter: 51.9 ± 5.8), 2) rats fed high-fat rat chow overnight and decapitated at the beginning of the light phase (plasma OXM-IR pmol/liter: 220.2 ± 22.2), and 3) rats fasted overnight, then given *ad libitum* access to high-fat chow for 2 h at lights-on, and decapitated at the end of the 2-h high-fat meal (plasma OXM-IR pmol/liter: 254.0 ± 32.7).
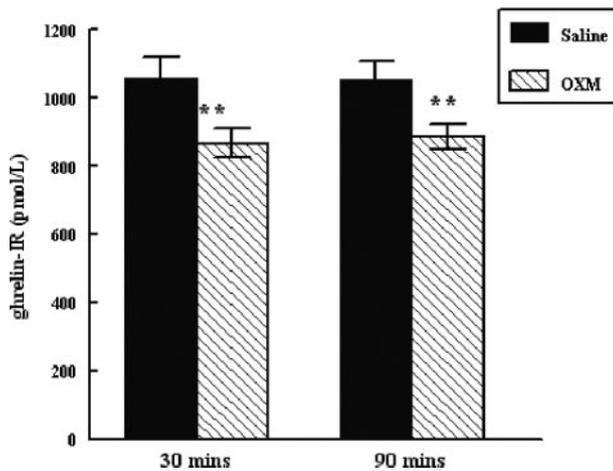
FIG. 6. The effect of ip administration of OXM (30 nmol/kg) or saline on plasma ghrelin-IR (pmol/liter). Rats were injected at time zero and decapitated 30 and 90 min post injection into overnight fasted rats (n = 10/group). **, $P < 0.01$ saline *vs.* OXM at each time point.

Intraperitoneal administration of OXM (30 and 100 nmol/kg) significantly decreased fasting plasma ghrelin-IR 30 and 90 min post injection [30-min plasma ghrelin picomoles/liter: saline, 1056.9 ± 64.0, OXM, 30 nmol/kg 867.4 ± 42.0 ($P < 0.01$), OXM, 100 nmol/kg 860.0 ± 47.5 ($P < 0.02$); 90-min plasma ghrelin-IR picomoles/liter: saline, 1055.2 ± 52.5, OXM, 30 nmol/kg 886.9 ± 36.3 ($P < 0.01$), OXM, 100 nmol/kg 900.0 ± 52.9 ($P < 0.05$)].

Plasma ghrelin-IR levels were determined in three additional groups: 1) rats fasted overnight and killed at the beginning of the light phase (plasma ghrelin-IR picomoles/liter: 1066.1 ± 80.9), 2) rats fed high-fat rat chow overnight and decapitated at the beginning of the light phase (plasma ghrelin-IR picomoles/liter: 611.3 ± 16.9), 3) rats fasted overnight, at lights-on given *ad libitum* access to high-fat chow for 2 h, and decapitated at the end of the 2-h high-fat meal (plasma ghrelin picomoles/liter: 648.9 ± 57.3).

Mapping the expression of FLI in the hypothalamus and brain stem in response to ip OXM

After ip OXM administration (50 nmol/kg), dense staining of FLI was found in the hypothalamic ARC (2.56 mm posterior to Bregma; OXM, 287 ± 10 counts *vs.* saline, 47 ± 5 counts; $P < 0.005$; Fig. 7A). No other hypothalamic nuclei (paraventricular nucleus, dorsomedial nucleus of the hypothalamus, ventromedial hypothalamic nucleus) demonstrated a specific increases in FLI (not shown).

In the brain stem (13.68 mm posterior to Bregma), ip CCK (15 nmol/kg) caused dense staining of FLI, most notably in the NTS and the area postrema. Neither ip saline nor OXM caused a specific increase in FLI in any other brain stem nuclei investigated (Fig. 7B).

Changes in αMSH and NPY release from hypothalamic explants when incubated with OXM

Incubating hypothalamic explants with OXM (100 nM) caused a significant increase in the release of αMSH, compared with basal release (αMSH: OXM, 100 nM, 16.4 ± 2.4 fmol/explant *vs.* basal 10.4 ± 2.0 fmol/explant; $P < 0.005$).

However, OXM did not affect the level of NPY release (NPY: OXM. 100 nM, 42.8 ± 15.6 fmol/explant *vs.* basal 46.8 ± 5.6 fmol/explant; $P = $ NS). In each case, explant viability was assessed by incubation with 56 mM KCl (and αMSH/NPY release measured). Viability was confirmed in more than 70% of explants. Nonviable explants were excluded from analyses.

Discussion

We found that peripheral administration of OXM caused a reduction in food intake in rats. OXM inhibited refeeding after a fast and during the nocturnal feeding phase. The anorectic effect was potent and sustained for up to 24 h. Twice-daily ip administration of OXM for 7 d caused a reduction in daily food intake, compared with those treated with saline, with no tachyphylaxis. Rats treated with OXM gained significantly less weight than pair-fed rats, despite the two groups receiving identical daily caloric intake. The related peptide, GLP-1, is known to reduce fluid intake (12, 14). However, although IP administration of OXM did transiently reduce water intake, this was not sustained, suggesting that the reduction in the rate of body weight gain was not due to dehydration. On conclusion of the chronic study, epididymal WAT and interscapular BAT were removed and weighed. It was found that there was a reduction in the weights of all fat pads in OXM-treated rats, compared with pair-fed rats. It is likely that, in addition to reducing daily food intake, peripheral OXM administration increases energy expenditure. Coupling of reduced appetite and increased energy expenditure has been observed previously with several hypothalamic systems (for review see Ref. 29).

Delayed gastric emptying can cause satiety via vagus-mediated mechanisms and the brain stem. Both GLP-1 and OXM are known to be inhibitors of gastric emptying in rodents and humans (7, 15, 30, 31) and in the case of GLP-1, this has been suggested to be a potential mechanism through which it promotes satiety. One hour post injection, the stomach contents of OXM-treated rats were greater than those of saline-treated controls, although this was not statistically significant ($P = 0.07$). However, no differences were observed at any of the later time points, suggesting a slight delay in gastric emptying may occur initially, but this is short lived and is unlikely to represent the primary mechanism by which OXM inhibits feeding.

Peripheral administration of OXM increased FLI almost exclusively in the ARC. Furthermore, incubating hypothalamic explants with OXM caused a significant increase in the release of the anorectic POMC-derived product, αMSH. There was no significant alteration in the release of orexigenic peptide, NPY. Intraperitoneal OXM did not affect the expression of FLI in the NTS and area postrema, areas known to be important in integrating vagally mediated information and a site of proglucagon expression and processing in the CNS, suggesting that OXM is not acting via these pathways.

Direct injection of OXM into the ARC at very low doses caused a robust and sustained inhibition of food intake. This effect is unlikely to be due to diffusion of the peptide into the ventricular system because the minimum effective intracerebroventricular dose of OXM causing an inhibition of food
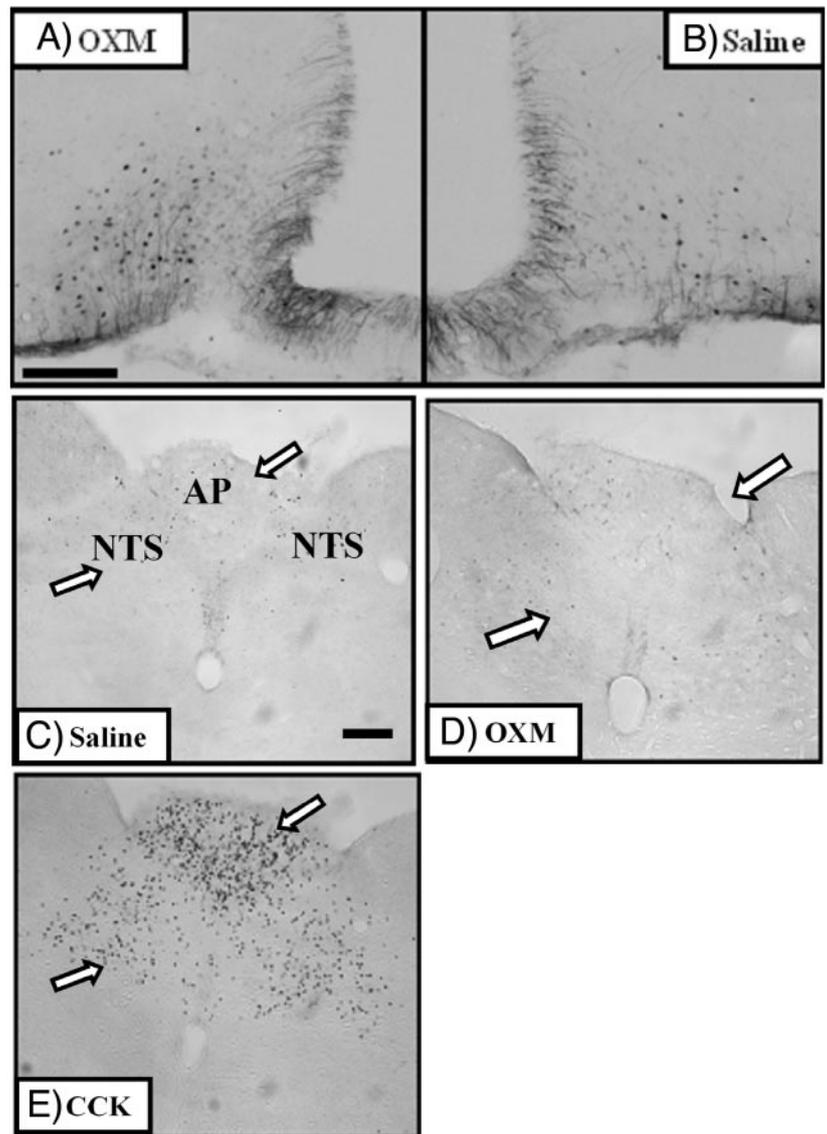
FIG. 7. Expression of FLI in response to ip administration of saline (A) or OXM (40 nmol/kg) (B) in a coronal section of the brain (40 μm) at the level of the hypothalamic ARC (*scale bar*, 200 μm) or ip administration of saline (C), OXM (50 nmol/kg) (D), or CCK (15 nmol/kg) in a coronal section of the brain stem (40 μm) at the level of the NTS and area postrema (AP) (*scale bar*, 200 μm; n = 8/group).

intake is 1 nmol (9). Thus, these doses of OXM are likely to be insufficient to elicit such a response, suggesting that the ARC is a site of action of OXM.

It is currently unclear through which receptor OXM mediates its actions. It has been reported that an OXM-specific binding site exists in the gastric mucosa (32). However, it has also been shown that the actions of OXM are mediated via a receptor, which also binds glicentin (33). Furthermore, it has been reported that OXM binds to the GLP-1 receptor and that it is the GLP-1 receptor at which OXM mediates its biological actions (34, 35).

Our receptor binding data have revealed that, in rat hypothalamic membrane preparations, the affinity of OXM for the GLP-1 receptor is approximately two orders of magnitude lower than GLP-1 (9). Therefore, it would be expected that 100 times greater concentrations of OXM would be required to elicit the same response as GLP-1 *in vivo*. We found that at the same doses, ip OXM is a more potent inhibitor of food intake than GLP-1, suggesting that OXM may not be acting through the cloned GLP-1 receptor. However, it

should be remembered that OXM is more stable than GLP-1 *in vivo*, and this could be reflected in its apparent enhanced potency. An alternative and currently untested possibility is that OXM is acting via a GLP-1 receptor whose ligand specificity is altered by an associated modifying protein, *i.e.* receptor activity modifying proteins (RAMPs).

The reduction in food intake observed after ip administration of OXM was blocked by prior administration of exendin$_{9-39}$ into the ARC. The anorectic actions of ip GLP-1, however, were not blocked. This surprising finding could suggest that GLP-1 and OXM act via distinct pathways. Furthermore, these findings might support the existence of an OXM-specific receptor in the ARC, to which exendin$_{9-39}$ also binds. However, conclusions drawn from experiments using exendin$_{9-39}$ are dependent on the specificity of exendin$_{9-39}$ as an antagonist. Serre *et al.* (36) reported that exendin$_{9-39}$ acts not only as an antagonist but also as an inverse agonist of the GLP-1 receptor. In addition, Malendowicz *et al.* (37) and Malendowicz and Nowak (38) published a series of experiments showing exendin$_{9-39}$-independent actions of GLP-1,

which they suggest cast doubt on the role of exendin$_{9-39}$ as a specific antagonist of the GLP-1 receptor. Further work needs to be performed to clarify the exact pathway and receptor through which OXM mediates its biological actions.

Thirty minutes post injection of OXM, plasma OXM-IR was significantly elevated when compared with the circulating levels observed postprandially. However, 90 min post injection, plasma OXM-IR was similar to that observed postprandially, and OXM was still actively inhibiting food intake at this time. Thirty minutes post injection, circulating levels of OXM-IR were comparable with the elevated concentrations seen in tropical sprue (39) and after jejunoileal bypass surgery (40, 41). OXM could contribute to the appetite and weight loss observed in these conditions. Intraperitoneal injections are known to cause a rapid rise in circulating plasma levels, and further investigations are required to determine the degree to which OXM inhibits food intake within the normal postprandial physiological range.

Ghrelin is a powerful stimulant of appetite in rodents and man (42), and preprandial rises in plasma ghrelin have been suggested to be a trigger for meal initiation (43). We found a significant suppression of fasting plasma ghrelin at both 30 and 90 min after peripheral OXM injection. If ghrelin is physiologically relevant, then the inhibition of fasting plasma ghrelin by OXM is a potential mechanism by which OXM reduces food intake. In further experiments we found that systemic administration of OXM significantly reduces food intake in healthy human subjects in a double-blind, cross-over study. Intravenous infusion of OXM reduced calorie intake by 19% in a buffet meal, and total caloric intake was decreased in the 12 h post infusion (44).

These data demonstrate that OXM can influence both long- and short-term regulation of food intake and body weight maintenance. These results suggest that circulating OXM could mediate its anorectic actions via direct interaction with the hypothalamus, activating POMC neurons within the ARC. In addition, peripheral OXM inhibits fasting plasma ghrelin. Therefore, OXM could offer a novel route for the development of therapeutic agents in the treatment of obesity.

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mation is fit for any particular purpose. The user thereof uses the information at its sole risk and liability.

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