

# Oxyntomodulin and Glucagon-Like Peptide-1 Differentially Regulate Murine Food Intake and Energy Expenditure

Laurie L. Baggio,\* Qingling Huang,\* Theodore J. Brown,<sup>†</sup> and Daniel J. Drucker\*

From the \*Department of Medicine, Banting and Best Diabetes Centre, Toronto General Hospital, University of Toronto, and the <sup>†</sup>Division of Reproductive Sciences, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, Ontario, Canada

**Background & Aims:** Gut-derived peptides including ghrelin, cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptide (GLP-1), and GLP-2 exert overlapping actions on energy homeostasis through defined G-protein-coupled receptors (GPCRs). The proglucagon-derived peptide (PGDP) oxyntomodulin (OXM) is cosecreted with GLP-1 and inhibits feeding in rodents and humans; however, a distinct receptor for OXM has not been identified. **Methods:** We examined the mechanisms mediating oxyntomodulin action using stable cell lines expressing specific PGDP receptors in vitro and both wild-type and knockout mice in vivo. **Results:** OXM activates signaling pathways in cells through glucagon or GLP-1 receptors (GLP-1R) but transiently inhibits food intake in vivo exclusively through the GLP-1R. Both OXM and the GLP-1R agonist exendin-4 (Ex-4) activated neuronal c-fos expression in the paraventricular nucleus of the hypothalamus, the area postrema, and the nucleus of the solitary tract following intraperitoneal (IP) injection. However, OXM transiently inhibited food intake in wild-type mice following intracerebroventricular (ICV) but not IP administration, whereas Ex-4 produced a more potent and sustained inhibition of food intake following both ICV and IP administration. The anorectic effects of OXM were preserved in *Gcgr*<sup>-/-</sup> mice but abolished in *GLP-1R*<sup>-/-</sup> mice. Although central Ex-4 and OXM inhibited feeding via a GLP-1R-dependent mechanism, Ex-4 but not OXM reduced  $\text{VO}_2$  and respiratory quotient in wild-type mice. **Conclusions:** These findings demonstrate that structurally distinct PGDPs differentially regulate food intake and energy expenditure by interacting with a GLP-1R-dependent pathway. Hence ligand-specific activation of a common GLP-1R increases the complexity of gut-central nervous system pathways regulating energy homeostasis and metabolic expenditure.

Peptide hormones released from enteroendocrine cells play an important role in regulating initiation of meal ingestion and satiety and the integrated control of energy homeostasis. Food intake promotes the rapid release of multiple gut peptides, which can activate ascending neural pathways or enter the central nervous system (CNS) directly, to terminate food ingestion. Hence, peptide hormones such as ghrelin, cholecystokinin (CCK),

and peptide YY (PYY) have been implicated as nutrient-sensitive modulators of feeding behavior, which exert their transient actions in part through control of gut motility and via communication with CNS satiety centers.<sup>1</sup> Furthermore, bariatric surgery is often associated with increased circulating levels of anorectic peptides released from the distal gut,<sup>2,3</sup> which may contribute to enhanced regulation of satiety, weight loss, and improved control of glucose homeostasis in selected patients.<sup>4</sup>

The proglucagon gene encodes structurally related proglucagon-derived peptides (PGDPs), including glucagon, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), glicentin, and oxyntomodulin (OXM). The intestinal PGDPs (Figure 1A) are rapidly secreted in response to food intake.<sup>5,6</sup> The actions of GLP-1 lower blood glucose through stimulation of glucose-dependent insulin secretion and inhibition of glucagon secretion and gastric emptying.<sup>7</sup> GLP-1 also inhibits food intake following peripheral administration, resulting in weight loss following administration to obese diabetic subjects.<sup>8</sup> In contrast, GLP-2 up-regulates intestinal hexose transport, inhibits gastric motility and acid secretion, and regulates the mass of the intestinal epithelium via stimulation of mucosal growth and inhibition of apoptosis.<sup>9</sup>

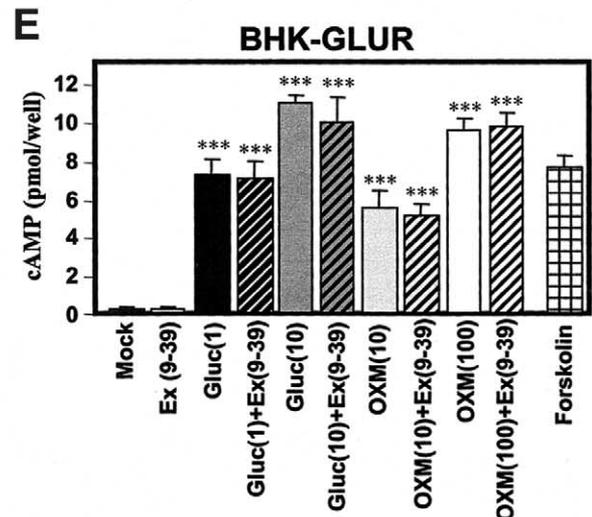
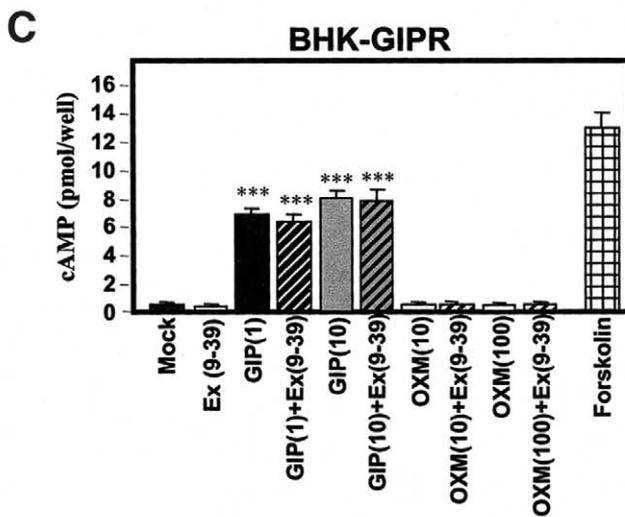
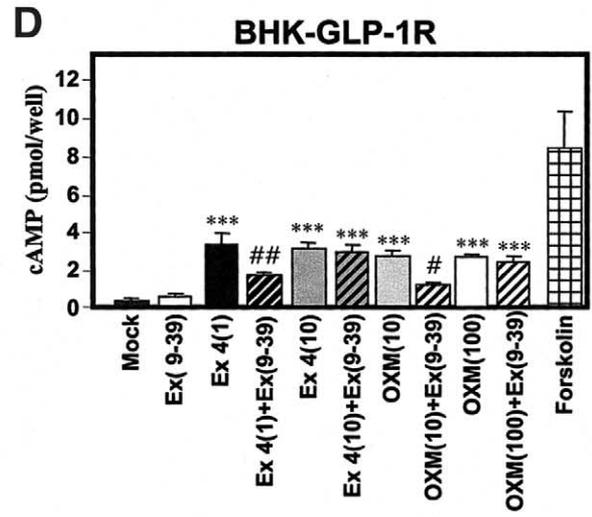
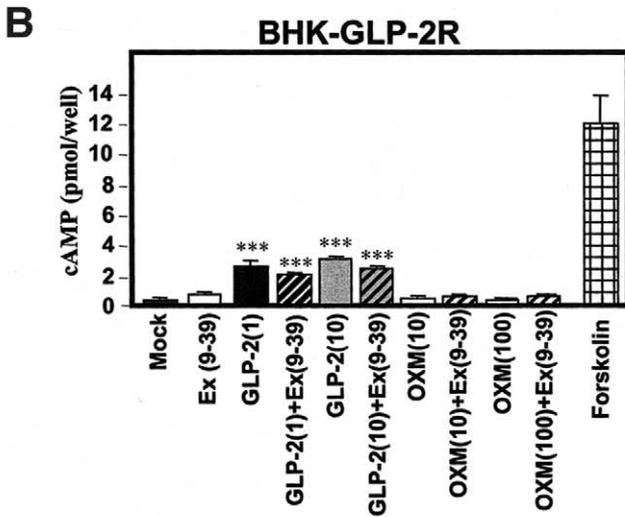
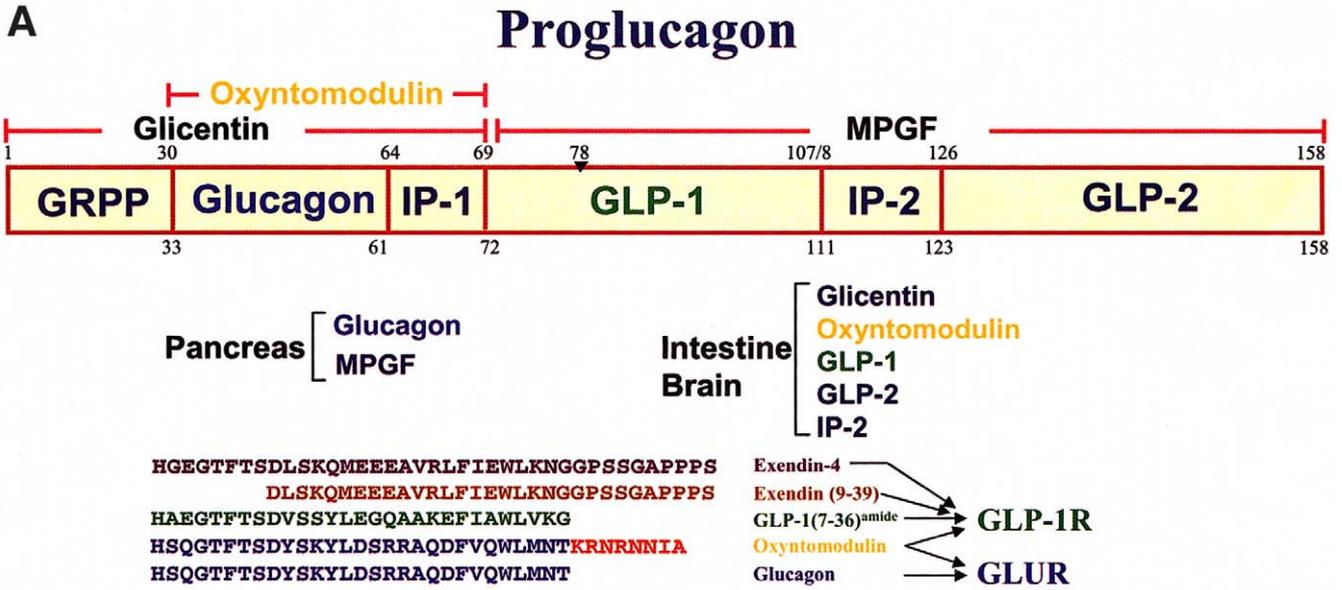
Proglucagon processing in the central nervous system generates a similar profile of PGDPs to that observed in the gut.<sup>10,11</sup> The CNS actions of GLP-1 are complex and include reduction of food intake, transduction of aversive signals, regulation of learning and memory, and neuroprotection.<sup>12,13</sup> Although much less is known about the physiologic importance of GLP-2 in the CNS, intracerebroventricular administration of GLP-2 inhibits food intake in both rats and mice.<sup>14,15</sup>

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**Abbreviations used in this paper:** CCK, cholecystokinin; Ex-4, exendin-4; GIPR, GIP receptor; GLP, glucagon-like peptide; GLP-1R, GLP-1 receptor; GLUR, glucagon receptor; GPCRs, G-protein-coupled receptors; OXM, oxyntomodulin; PGDP, proglucagon-derived peptide; PYY, peptide YY.

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In contrast to the expanding number of actions described for GLP-1 and GLP-2, the biologic activities of circulating PGDPs such as gut-derived glicentin and OXM are much less understood. Glicentin, a 69 amino acid peptide containing the sequence of OXM, exhibits modest intestinotrophic effects.<sup>16,17</sup> OXM, a 37 amino acid peptide containing the sequence of glucagon and an 8 amino acid carboxyterminal extension (Figure 1A), inhibits gastric emptying and gastric acid secretion in both rodents and humans<sup>18,19</sup> and stimulates intestinal glucose uptake and decreases pancreatic enzyme secretion in rats.<sup>20,21</sup> Central administration of OXM reduces food intake in both fasted and fed rats,<sup>22</sup> whereas twice daily intracerebroventricular (ICV) or intraperitoneal (IP) injections of OXM decreased both food intake and body weight gain.<sup>23,24</sup> Furthermore, OXM inhibits food intake following parenteral administration to normal human subjects.<sup>25</sup>

Although separate receptors have been characterized for glucagon, GLP-1, and GLP-2,<sup>26</sup> pharmacologically unique binding sites or distinct receptors for glicentin or OXM have not yet been identified. Furthermore, selective antagonists that specifically block the actions of OXM are not available. Although the truncated lizard peptide exendin (9-39) (Figure 1A) inhibits the anorectic actions of OXM, exendin (9-39) has been shown to function either as a GLP-1, glucose-dependent insulinotropic peptide (GIP), or GLP-2 receptor antagonist.<sup>14,27,28</sup> OXM binds to porcine glucagon receptors, albeit with much lower affinity compared to glucagon<sup>29</sup>; however, OXM can also bind to and activate GLP-1 receptors, increasing cAMP accumulation and stimulating somatostatin secretion and H<sup>+</sup> production in RIN T3 and rat parietal cell preparations, respectively.<sup>30,31</sup> Hence, the precise molecular mechanisms whereby OXM mediates its biologic actions, including inhibition of food intake, remain inconclusive.<sup>22</sup>

The available data suggest that enteroendocrine-derived peptides exert distinct biologic actions through single unique receptors; hence, the possibility that 2 structurally related PGDPs, GLP-1 and OXM, may exert overlapping biologic actions via a single common recep-

tor has not yet been clearly established. OXM potentially interacts with either the glucagon or GLP-1 receptors, and GLP-2-dependent modulation of OXM activity has also been described.<sup>30-32</sup> Furthermore, the mechanism by which OXM produces weight loss in rodents has not been determined, and an effect of OXM on ghrelin secretion and/or energy expenditure has been postulated.<sup>23,24</sup> Accordingly, to identify the specific receptor(s) transducing the biologic effects of OXM, we utilized a combination of cell lines and both wild-type and receptor knockout mice for analysis of ligand binding, signal transduction, food intake, neuronal activation, and energy expenditure. The results of these studies provide new evidence that 2 distinct peptide agonists derived from the same proglucagon gene activate a single GLP-1–preferring receptor, yet exhibit divergent effects on food intake vs. control of energy expenditure.

## Materials and Methods

### Reagents

Tissue culture medium, serum, and G418 were purchased from Invitrogen (San Diego, CA). Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO). OXM and exendin (9-39) were initially obtained from Dr. Stephen Bloom (Dept. of Metabolic Medicine, Imperial College, Hammersmith Hospital, London, United Kingdom) and were subsequently purchased separately from California Peptide Research Inc. (Napa, CA). Exendin-4 (Ex-4) was obtained from California Peptide Research Inc. (Napa, CA), and human GLP-2 was provided by Bachem Inc. (Torrance, CA).

### Cell Culture and Transfections

Baby hamster kidney (BHK) fibroblast cells were transfected with cDNA encoding either the rat GIP receptor (GIPR)<sup>33</sup> or the rat glucagon receptor (GLUR)<sup>34</sup> cloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA) or with pcDNA3.1 alone using Lipofectin reagent (Invitrogen), according to the manufacturer's protocol. Stable pcDNA3.1-, GIPR-, or GLUR-expressing cell lines were generated by selection of transfected cells in medium supplemented with G418 (0.8 mg/mL) for 2 weeks. Cell culture studies were carried out using pools of stable G418-resistant

**Figure 1.** Oxyntomodulin stimulates cAMP production via the GLP-1 and glucagon receptors in vitro. (A) Structure of proglucagon and the proglucagon-derived peptides (PGDPs) including oxyntomodulin. The amino acid sequences of exendin-4, exendin (9-39), GLP-1, oxyntomodulin, and glucagon are shown below. GLP-1R, glucagon-like peptide-1 receptor; GLUR, glucagon receptor; GRPP, glicentin-related pancreatic polypeptide; MPPGF, major proglucagon fragment; IP, intervening peptide. (B–E) Stable BHK cell lines expressing rat receptors for GLP-2 (B), GIP (C), GLP-1 (D), or glucagon (E) were pretreated for 5 minutes with 500 nmol/L exendin (9-39) [Ex9-39] or medium alone prior to a 10-minute treatment with 1 nmol/L (1), 10 nmol/L (10), or 100 nmol/L (100) of GLP-2, oxyntomodulin (OXM), GIP, exendin-4 (Ex 4), or glucagon (gluc). The cAMP concentration in aliquots of lyophilized cell extracts was determined by radioimmunoassay and used to calculate the total cAMP content per well. Forskolin treatment (100 μmol/L) was used as a positive control. Values are expressed as means ± SD and are representative of data from 2 independent experiments, each performed in triplicate. \*\*\**P* < 0.001 vs. mock; #*P* < 0.05, ##*P* < 0.01, for peptide- vs. exendin (9-39)-treated cells.

clones maintained in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L glucose) supplemented with 10% fetal bovine serum and 0.05 mg/mL G418. BHK cell lines transfected with either the rat GLP-1R or the rat GLP-2R were generated and propagated as described previously.<sup>35,36</sup>

### Cell-Based Receptor Activation Studies

BHK cells stably expressing either pcDNA 3.1, GLP-1R, GLP-2R, or GIPR or the GLUR were grown to 70%–80% confluence in 24-well plates in the absence of G418 at 37°C. All peptides were initially dissolved in phosphate-buffered saline (PBS), and cells were treated with individual peptides or 100  $\mu$ M forskolin in DMEM containing serum and 100  $\mu$ M IBMX. For peptide treatments, cells were incubated with exendin (9–39) or medium alone for 5 minutes at 37°C, followed by an additional 10-minute incubation in the presence of different concentrations of Ex-4, GIP, GLP-1, GLP-2, or glucagon. All reactions were carried out in triplicate and terminated by the addition of ice-cold absolute ethanol. Cell extracts were collected and stored at  $-80^{\circ}\text{C}$  until assayed. For cAMP determinations, aliquots of ethanol extracts were lyophilized, and cAMP levels were measured using a cAMP radioimmunoassay kit (Biomedical Technologies, Stoughton, MA).

### Animal Experiments

All animal experiments were carried out in accordance with protocols and guidelines approved by the Toronto General Hospital Animal Care Committee. Mice were maintained under a 12-hour light/dark cycle and allowed free access to standard rodent chow and water, except where noted. The generation and characterization of GLP-1R<sup>-/-</sup> mice in the CD-1 genetic background has been described.<sup>37–39</sup> GLUR<sup>-/-</sup> mice (Gcgr<sup>-/-</sup>) in the C57BL/6 genetic background were kindly provided by Dr. Erica Nishimura (Dept. of Diabetes Biology, Novo Nordisk A/S, Bagsvaerd, Denmark) and Dr. Maureen Charron (Dept. of Biochemistry, Albert Einstein College of Medicine, Bronx, NY). Age- and sex-matched (14- to 16-week-old males) wild-type CD-1 or C57BL/6 control mice were obtained from Charles River Laboratories (Montreal, Quebec, Canada) and were allowed to acclimatize to the animal facility for a minimum of 1 week.

For analysis of food intake, mice were fasted overnight (16–18 hours), and peptides (dissolved in PBS) or vehicle (PBS) were administered by either IP or ICV injection. For ICV injections, mice were lightly anesthetized using isoflurane inhalation (Abbott Laboratories Ltd., Saint-Laurent, Quebec, Canada), and peptides or vehicle were administered in a total volume of 5  $\mu$ L by injection into the lateral ventricles using a 2.5-mm, 30-gauge needle attached to a Hamilton syringe as described.<sup>37</sup> Mice were allowed to recover from the anesthetic (approximately 10 minutes) prior to assessment of food intake. For IP injections, 100  $\mu$ L of peptide or vehicle were injected into the peritoneal cavity. Following peptide administration, mice were weighed and then placed into individual cages containing preweighed rodent chow, with free access to water.

At 2, 4, 8, and 24 hours following peptide or vehicle administration, the chow was reweighed, and total food intake (g/g of body weight) was calculated.

### Indirect Calorimetry

Immediately following IP or ICV administration of PBS or peptide, oxygen consumption ( $\text{VO}_2$ ) and carbon dioxide generation ( $\text{VCO}_2$ ) were determined by indirect calorimetry using an Oxymax System (Columbus Instruments, Columbus, OH). Mice were placed into individual metabolic chambers with free access to food and water.  $\text{VO}_2$  and  $\text{VCO}_2$  were measured, and respiratory exchange ratio (RER; ratio of  $\text{VO}_2$  to  $\text{VCO}_2$ ) was determined at 15-minute intervals for a total of 21 hours.

### Assessment of *c-fos* Activation in the Murine Central Nervous System

The number of *c-fos*-immunoreactive neurons in specific brain regions was quantitatively assessed in both wild-type CD-1 and GLP-1R<sup>-/-</sup> mice as described.<sup>40,41</sup> Briefly, animals were administered an IP injection of PBS or peptide in a 100  $\mu$ L volume. At 60 minutes following injection, mice were anesthetized with Somnotol (sodium pentobarbital solution, MTC Pharmaceuticals, Cambridge, Ontario, Canada). All mice were perfused intracardially with ice-cold normal saline followed by 4% paraformaldehyde solution. Brains were removed immediately at the end of perfusion and kept in ice-cold 4% paraformaldehyde solution for 3 days then transferred to a solution containing paraformaldehyde and 10% sucrose for 12 hours. Brains were cut into 25- $\mu$ m sections using a sliding microtome Leica SM2000R (Leica Microsystems, Richmond Hill, Ontario, Canada) and stored at  $-30^{\circ}\text{C}$  in a cold cryoprotecting solution. Sections were processed for immunocytochemical detection of Fos using a conventional avidin-biotin-immunoperoxidase method (Vectastain ABC Elite Kit; Vector Laboratories, CA) as described.<sup>40,41</sup> The Fos antibody (Sigma-Aldrich, Oakville, ON) was used at a 1:50,000 dilution. Brain sections corresponding to the level of the arcuate nucleus (ARC), the medial parvocellular portion of the paraventricular nucleus of the hypothalamus (PVH), the nucleus of solitary tract (NTS), and the area postrema (AP) were selected for analyses. Sections corresponding to  $-1.58$  mm to  $1.94$  mm (ARC),  $-0.70$  mm to  $-1.94$  mm (PVH), and  $-7.48$  mm (NTS and AP) from bregma, respectively, were defined according to the Mouse Brain Atlas of Franklin and Paxinos.<sup>42</sup>

### GLP-1 Binding Studies

<sup>125</sup>I-labeled GLP-1 and <sup>125</sup>I-labeled oxyntomodulin (specific activity 898 and 868 Ci/mmol, respectively) were purchased from Peninsula Laboratories (San Carlos, CA). Frozen coronal brain sections (16  $\mu$ m) were thaw mounted onto slides and preincubated for 20 minutes at room temperature in assay buffer [25 mmol/L HEPES buffer (pH 7.4) containing 2 mmol/L  $\text{MgCl}_2$ , 1% BSA, 1 mmol/L dithiothreitol, 0.05% Tween 20, and 0.1% bacitracin]. Sections were incubated for 90 minutes at room temperature in assay buffer containing 60

pmol/L  $^{125}\text{I}$ -labeled GLP-1 or 120 pmol/L  $^{125}\text{I}$ -labeled oxyntomodulin alone or in the presence of 200 nmol/L unlabeled GLP-1, oxyntomodulin, or exendin (9-39). Slides were rinsed 3 times for 1 minute each in assay buffer at 4°C, dipped in ice-cold distilled water, and then dried overnight under an air stream at 4°C. Slides were exposed to film (Kodak BioMax MR) at room temperature for 10 days.

### Statistical Analysis

All data are presented as means  $\pm$  SD or SE as indicated. Statistical significance was determined by ANOVA and Bonferonni's posttest using Prism Version 3.03 software (GraphPad Software Inc., San Diego, CA) or by ANOVA followed by Fisher post hoc analysis using StatView (Abacus, CA). A  $P$  value  $< 0.05$  was considered to be statistically significant.

### Results

To identify the specific receptor(s) through which OXM mediates its effects, we examined the ability of OXM to stimulate cAMP formation in stable clones of BHK fibroblast cells expressing either the rat GLP-2, GIP, GLP-1, or glucagon receptor. In BHK cells expressing the GLUR, GLP-2R, or GIPR, treatment with 1 or 10 nmol/L glucagon, GLP-2, or GIP, respectively, elicited a significant rise in cAMP (Figure 1E, 1B, and 1C, respectively); the stimulatory action of these peptides on their known receptors was not inhibited by the GLP-1 receptor antagonist exendin (9-39) (Figure 1E, 1B, and 1C, respectively). Treatment of BHK-GIPR or BHK-GLP-2R cells with OXM (10 or 100 nmol/L) had no effect on cAMP production (Figure 1C and 1B, respectively). Conversely, BHK cells that express the rat GLP-1 or glucagon receptors exhibited significant increases in cAMP accumulation in response to treatment with either their cognate ligand or OXM (Figure 1D and E). Moreover, pretreatment of BHK-GLP-1R cells with exendin (9-39) significantly attenuated the increase in cAMP produced in response to the lower concentrations of the GLP-1 receptor agonist Ex-4 (1 nmol/L) or OXM (10 nmol/L) (Figure 1D). In contrast, pretreatment of BHK-GLUR cells with exendin (9-39) had no inhibitory effect on cAMP production in response to glucagon or OXM (Figure 1E). These results demonstrate that (1) OXM activates both the cloned glucagon and GLP-1 receptors and that (2) exendin (9-39) is a functional OXM antagonist at the rat GLP-1 but not the glucagon receptor in vitro.

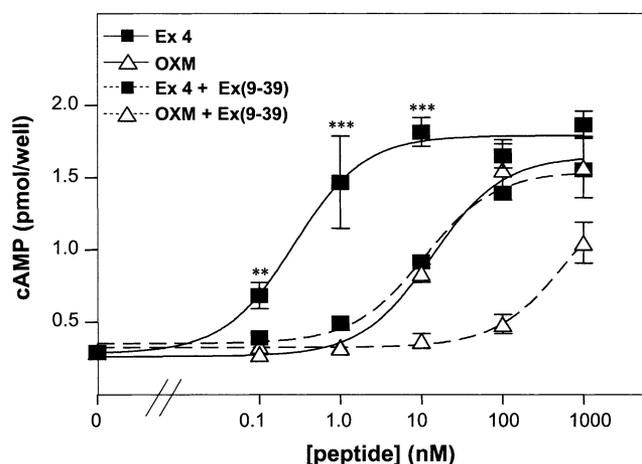
### OXM Is a Less Potent GLP-1R Agonist Compared With Ex-4

The results of experiments in Figure 1D demonstrate that OXM can function as a GLP-1R agonist,

potentially in an exendin (9-39)-dependent manner. To determine the relative potency of OXM vs. Ex-4 at the GLP-1 receptor, we incubated BHK-GLP-1R fibroblasts with increasing concentrations of Ex-4 or OXM in the presence or absence of 1  $\mu\text{mol/L}$  exendin (9-39). Although both Ex-4 and OXM increased levels of cAMP in a dose-dependent manner, Ex-4 was significantly more potent than OXM over a range of peptide concentrations (Figure 2;  $\text{EC}_{50} = 0.27$  nmol/L for Ex-4 vs. 13.69 nmol/L for OXM), demonstrating that OXM is a comparatively weak agonist at the rat GLP-1R. Pretreatment with 1  $\mu\text{mol/L}$  exendin (9-39) diminished both the Ex-4- and OXM-stimulated elevations in cAMP (Figure 2);  $\text{EC}_{50} = 10.93$  nmol/L for Ex-4 + exendin (9-39) and 0.61  $\mu\text{mol/L}$  for OXM + exendin (9-39). In contrast, treatment with exendin (9-39) alone had no effect on levels of cAMP in BHK-GLP-1R cells (data not shown). Furthermore, the data demonstrate that a 100-fold molar excess of exendin (9-39) is required to block the actions of Ex-4 in vitro.

### ICV OXM Inhibits Food Intake in a Dose-Dependent Manner in Wild-Type Mice

GLP-1, Ex-4, and, more recently, OXM have been shown to significantly reduce food intake in rodents and humans.<sup>43-45</sup> To identify the receptor(s) transducing the anorectic effects of OXM, we administered OXM



**Figure 2.** Oxyntomodulin is a less potent agonist of the rat GLP-1 receptor than exendin-4 in vitro. A stable BHK cell line expressing the rat GLP-1 receptor (GLP-1R) was pretreated for 5 minutes with 1  $\mu\text{mol/L}$  exendin (9-39) or medium alone prior to a 10-minute treatment with increasing concentrations of exendin-4 (Ex 4) or oxyntomodulin (OXM). cAMP levels in lyophilized aliquots of cell extracts were quantified by radioimmunoassay and used to calculate the total cAMP content per well. Values are expressed as means  $\pm$  SD and are representative of data from 2 independent experiments, each performed in triplicate. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for Ex 4- vs. OXM-treated cells.

(1–15  $\mu\text{g}$ ) by ICV injection to wild-type mice. OXM, at ICV doses ranging from 5 to 15  $\mu\text{g}$ , significantly reduced feeding in wild-type mice for at least 8 hours; however, by 24 hours, food intake was not different from that of control (PBS)-treated mice (Figure 3A). A lower dose (1  $\mu\text{g}$ ) of ICV OXM had no effect on food consumption in wild-type mice (Figure 3A). In contrast, ICV administration of 1  $\mu\text{g}$  Ex-4 resulted in a sustained reduction in food intake over the 24-hour observation period (Figure 3A). Thus, the inhibitory effect of ICV OXM on food intake in mice is dose dependent but is less potent and more transient relative to that of the GLP-1R agonist Ex-4.

### ICV OXM Inhibits Food Intake in an Exendin (9-39)-Dependent Manner

Exendin (9-39) inhibited the anorectic effect of both ICV OXM and GLP-2 in rats<sup>14,22</sup>; hence, the receptor and species specificity of exendin (9-39) as a PGDP receptor antagonist has been questioned because exendin (9-39) does not inhibit and actually potentiates the anorectic effects of ICV GLP-2 in wild-type mice.<sup>15</sup> Central administration of exendin (9-39) completely blocked the inhibitory effects of ICV OXM on food intake and partially reversed the inhibitory effect of Ex-4 on food ingestion (Figure 3B). These data demonstrate that exendin (9-39) is also a functional OXM antagonist in wild-type mice.

### ICV OXM Does Not Inhibit Food Intake in GLP-1R<sup>-/-</sup> Mice

The results of several studies allude to the possible existence of a second GLP-1R in adipocytes, hepatocytes, and myocytes that is distinct from the known cloned pancreatic GLP-1 receptor.<sup>46,47</sup> Furthermore, other studies demonstrate a lack of an inhibitory effect of exendin (9-39) on GLP-1 action in the liver and gastrointestinal tract.<sup>48,49</sup> Thus, OXM or, in some instances, Ex-4 might potentially exert actions via a distinct receptor structurally similar to the known GLP-1R. Nevertheless, ICV administration of OXM or Ex-4, at doses that inhibit food intake in wild-type mice, had no effect on feeding in GLP-1R<sup>-/-</sup> mice (Figure 3C), demonstrating that OXM and Ex-4 modulate feeding behavior exclusively through the known defined GLP-1R.<sup>50</sup>

### IP Administration of OXM Does Not Inhibit Food Intake in Wild-Type or GLP-1R<sup>-/-</sup> Mice

Peripheral administration of GLP-1R agonists reduced food intake in both rodents and humans.<sup>44,45,51</sup>

Similarly, IP OXM inhibited food intake and body weight gain in rats,<sup>24</sup> and intravenous administration of OXM increased satiety and reduced caloric intake in humans.<sup>25</sup> Nevertheless, peripheral administration of OXM, even at pharmacologic doses (15–100  $\mu\text{g}$ ), had no effect on food intake in wild-type mice (Figure 3D). In contrast, a much smaller IP dose of Ex-4 (1.5  $\mu\text{g}$ ) significantly reduced food intake for 24 hours (Figure 3D). These results suggest the existence of species-specific differences in peripherally accessible Ex-4- vs. OXM-sensitive pathways modulating feeding behavior.

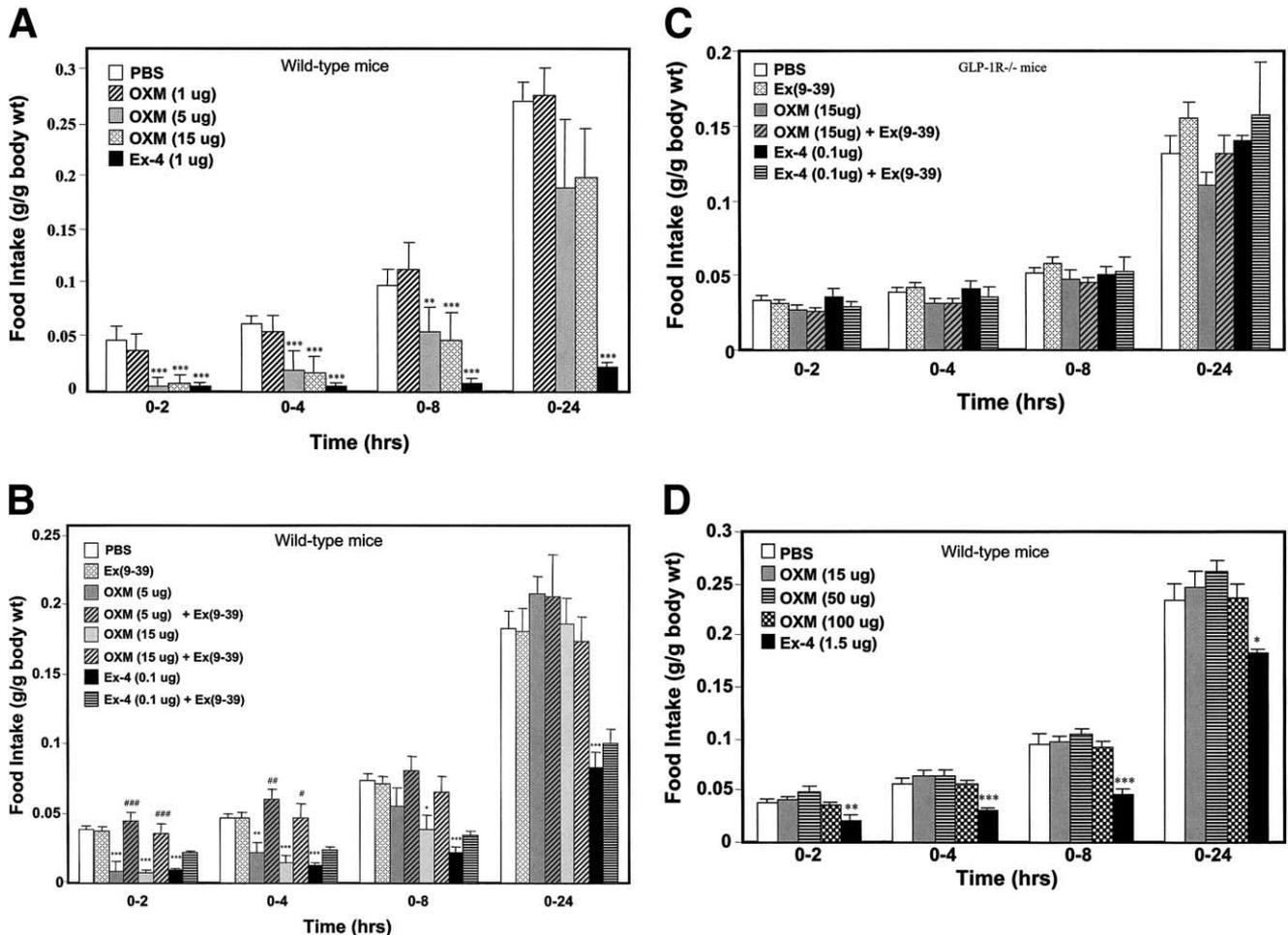
GLP-1R<sup>-/-</sup> mice exhibit enhanced sensitivity to the anorectic effects of GLP-2,<sup>15</sup> suggesting that the presence or absence of the GLP-1R functionally modulates activity of feeding pathways regulated by structurally related PGDPs. To determine whether potential anorectic effects of peripheral OXM could be mediated by a separate receptor unmasked by absence of the known GLP-1R, we measured food intake in GLP-1R<sup>-/-</sup> mice. IP injection of either OXM or Ex-4 had no effect on feeding in GLP-1R<sup>-/-</sup> mice (Figure 3E). Taken together, the data obtained using both wild-type and GLP-1R<sup>-/-</sup> animals demonstrate that, at the doses tested, peripherally administered OXM does not modulate feeding behavior in mice.

### ICV OXM Inhibits Food Intake in Gcgr<sup>-/-</sup> Mice

OXM interacts with the liver glucagon receptor,<sup>29</sup> and our experiments with the cloned glucagon receptor expressed in BHK cells demonstrate that OXM is capable of activating signaling pathways through the Gcgr (Figure 1E). To determine whether the Gcgr is also required for the inhibitory effect of OXM on feeding in vivo, we measured food intake in fasting Gcgr<sup>-/-</sup> mice in response to ICV OXM. Because the Gcgr<sup>-/-</sup> mice are maintained on the C57BL/6 genetic background, we also measured food intake in wild-type C57BL/6 mice. OXM significantly decreased feeding in both wild-type C57BL/6 (data not shown) and Gcgr<sup>-/-</sup> mice (Figure 3F), demonstrating that the anorectic effects of OXM do not require a functional glucagon receptor.

### OXM and Ex-4 Activate c-fos in the Murine Brain

The observation that Ex-4 but not OXM inhibited food intake after IP administration prompted us to ascertain whether peripheral administration of different GLP-1R agonists produced comparable activation of CNS regulatory circuits implicated in the control of food intake. Ex-4 significantly induced neuronal *c-fos* expression in the paraventricular nucleus of the hypothalamus, the nucleus of the



**Figure 3.** (A) ICV oxyntomodulin inhibits food intake in a dose-dependent manner in wild-type mice. Following an overnight (16–18 hour) fast, wild-type CD-1 mice were anesthetized and given intracerebroventricular (ICV) injections of vehicle (PBS) or increasing doses of oxyntomodulin (OXM) or exendin-4 (Ex-4). Food intake was measured at 2, 4, 8, and 24 hours postrecovery from injection. Values are expressed as means  $\pm$  SE;  $n = 4$ –7 mice/treatment.  $**P < 0.01$ ,  $***P < 0.001$  for PBS- vs. peptide-treated mice. (B) The inhibitory effect of ICV oxyntomodulin in wild-type mice is blocked by coadministration of exendin (9-39). Wild-type CD-1 mice were fasted overnight (16–18 hours) and the following day were given intracerebroventricular (ICV) injections of either vehicle (PBS), oxyntomodulin (OXM), or exendin-4 (Ex-4), in the presence or absence of 50  $\mu$ g exendin (9-39) [Ex(9-39)]. Food intake was measured at 2, 4, 8, and 24 hours post-recovery from injection. Values are expressed as means  $\pm$  SE and contain data from several independent experiments;  $n = 4$ –20 mice/treatment.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  for PBS- vs. peptide-treated mice;  $\#P < 0.05$ ,  $\#\#P < 0.01$ ,  $\#\#\#P < 0.001$  for peptide- vs. exendin (9-39)-treated mice. (C) ICV oxyntomodulin has no effect on food intake in GLP-1R<sup>-/-</sup> mice. Following an overnight fast (16–18 hours), GLP-1R<sup>-/-</sup> mice were anesthetized and given intracerebroventricular (ICV) injections of either vehicle (PBS), oxyntomodulin (OXM), or exendin-4 (Ex-4), in the presence or absence of 50  $\mu$ g exendin (9-39) (Ex9-39). Food intake was measured at 2, 4, 8, and 24 hours postrecovery from injection. Values are expressed as means  $\pm$  SE;  $n = 5$ –12 mice/treatment. (D and E) IP oxyntomodulin has no effect on food intake in either wild-type or GLP-1R<sup>-/-</sup> mice. After a 16–18-hour overnight fast, wild-type CD-1 (D) or GLP-1R<sup>-/-</sup> (E) mice were given intraperitoneal (IP) injections of vehicle (PBS), oxyntomodulin (OXM), or exendin-4 (Ex-4). Food intake was measured at 2, 4, 8, and 24 hours postinjection. Values are expressed as means  $\pm$  SE;  $n = 5$ –8 mice/treatment.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  for PBS- vs. peptide-treated mice. (F) ICV oxyntomodulin inhibits food intake in Gcgr<sup>-/-</sup> mice. Gcgr<sup>-/-</sup> mice were fasted overnight (16 hours), and the following day, they were lightly anesthetized and given intracerebroventricular (ICV) injections of either vehicle (PBS), oxyntomodulin (OXM), or exendin-4 (Ex-4). Food intake was measured at 2, 4, 7 (for technical reasons, 7 and not 8 hours was selected for this study), and 24 hours postrecovery from injection. Values are expressed as means  $\pm$  SE;  $n = 3$  mice/treatment.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  for PBS- vs. peptide-treated mice. Figure 3 continued on facing page.

solitary tract, and the area postrema but not in the arcuate nucleus of wild-type mice (Figure 4). Similarly, IP OXM administration produced significant *c-fos* activation in the same brain regions (Figure 4). In contrast, both peptides failed to increase *c-fos* expression following IP administration in GLP-1R<sup>-/-</sup> mice (Figure 4).

### Acute Administration of Ex-4 but not OXM Regulates Metabolic Rate in Mice

Although considerable data support an acute inhibitory effect of specific PGDPs on food ingestion, whether these anorectic peptides also regulate metabolic

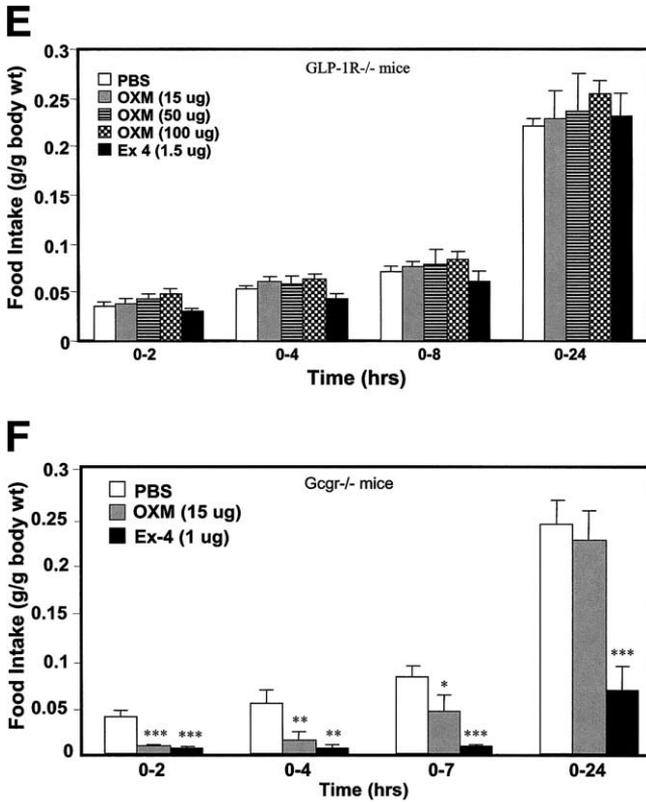


Figure 3. (Cont'd.)

rate has not been extensively examined. Intracerebroventricular or intraperitoneal administration of OXM in wild-type mice had no effect on  $VO_2$  or the respiratory exchange ratio (RER), either immediately following peptide injection or in the 21-hour period after OXM administration (Figure 5 and data not shown). In contrast, ICV administration of Ex-4 produced a rapid decrease in  $VO_2$ , which was associated with a significant reduction in RER over the 21-hour observation period (Figure 5). Similarly, IP administration of Ex-4, significantly ( $P < 0.01$  Ex-4 vs. control) reduced  $VO_2$ , and RER, but the effect was more transient and limited to the first 2 hours after peptide administration (data not shown).

### OXM Competes for GLP-1 Binding to the CNS GLP-1 Receptor

To determine whether GLP-1 and OXM function as competitive peptide ligands at the GLP-1 receptor, we carried out binding studies using [<sup>125</sup>I]-labeled GLP-1 and sections of the murine central nervous system (Figure 6). Discrete binding of radiolabeled GLP-1 was detected in several regions including the central amygdala, dentate gyrus, medial and lateral habenular nucleus, medio-basal and lateral hypothalamus, and the zona incerta (Figure 6A). [<sup>125</sup>I]-labeled GLP-1 binding was completely blocked in the presence of unlabeled GLP-1, or the antagonist exendin (9-39), and was markedly dimin-

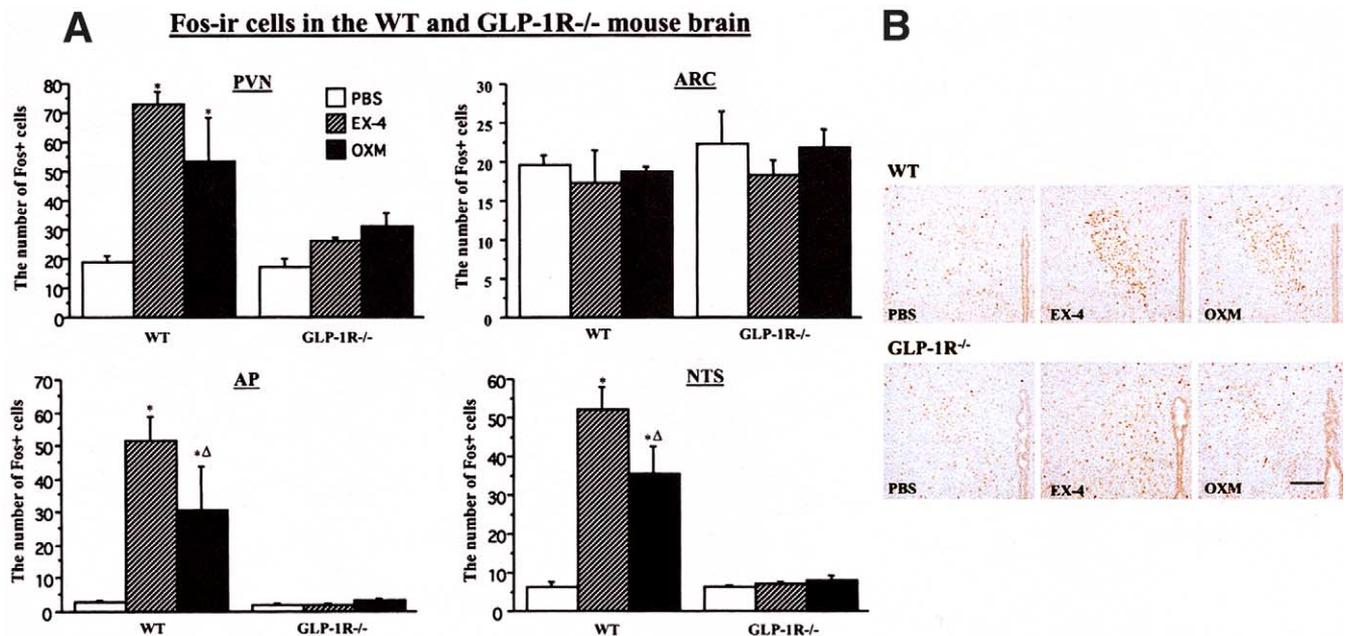
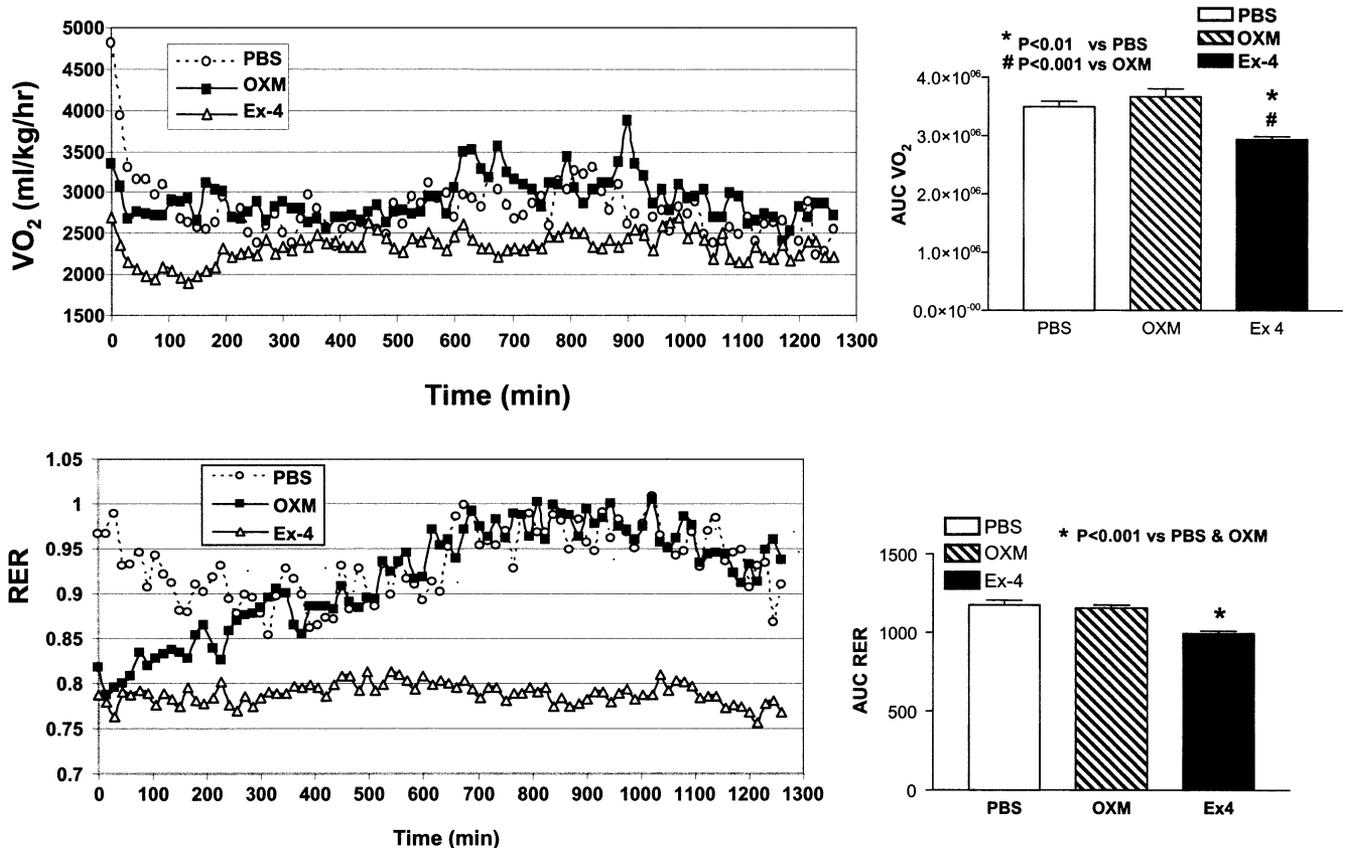


Figure 4. IP OXM and Ex-4 stimulate neuronal activity in the murine CNS. (A) The number of *c-fos* immunopositive cells (*Fos*<sup>+</sup>) in the paraventricular nucleus of the hypothalamus (PVH), arcuate nucleus (ARC), area postrema (AP), and the nucleus of solitary tract (NTS) and (B) representative photomicrographs of *Fos*-stained coronal brain sections of the hypothalamic paraventricular nucleus (PVH) from wild-type (WT) and GLP-1R<sup>-/-</sup> mice at 60 minutes following IP administration of vehicle (PBS), exendin-4 (EX-4; 1.5 μg), or oxyntomodulin (OXM; 50 μg). Data are represented as means ± SE; n = 4 mice/treatment. \* $P < 0.01$  experimental vs. control; Δ $P < 0.01$ , OXM vs. Ex-4; Bar = 200 μm. Statistical significance between groups was calculated by ANOVA followed by Fisher post hoc analysis.



**Figure 5.** ICV administration of Ex-4 but not OXM modifies oxygen consumption and RER in mice. Wild-type CD-1 mice were anesthetized and given intracerebroventricular injections of vehicle (PBS), oxyntomodulin (OXM; 5  $\mu$ g), or exendin-4 (Ex-4; 1  $\mu$ g) in a total volume of 5  $\mu$ L. Immediately following peptide administration, mice were placed into individual metabolic chambers and oxygen consumption ( $VO_2$ ) and respiratory exchange ratio (RER) were measured at 15-minute intervals for a total of 21 hours.

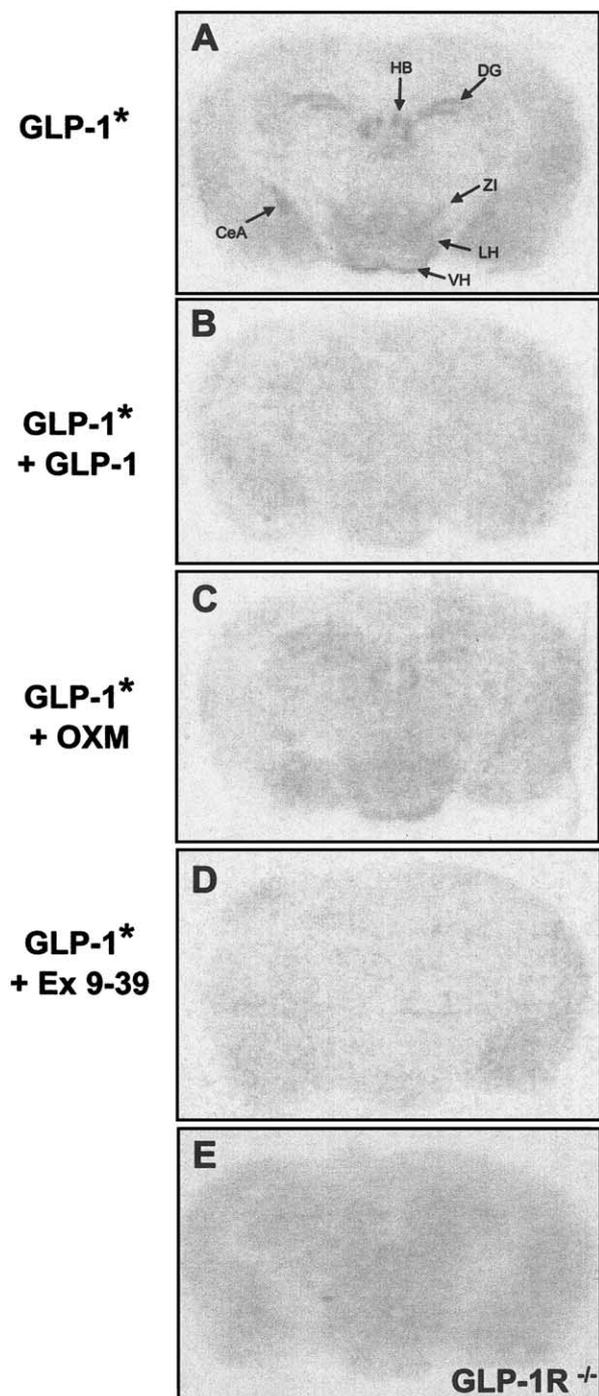
ished by unlabeled oxyntomodulin (Figure 6B–D). In contrast, no detectable binding of [<sup>125</sup>I]-labeled GLP-1 was observed using coronal sections from GLP-1R<sup>-/-</sup> mice (Figure 6E). Similarly, much weaker binding of [<sup>125</sup>I]-labeled oxyntomodulin was detected in the arcuate and supraoptic nucleus using identical coronal sections from wild-type but not GLP-1R<sup>-/-</sup> mice (data not shown).

## Discussion

The concept that 2 distinct peptides modulate biologic actions through a shared common receptor appears increasingly likely. For example, PTH and PTHrP compete for binding at the PTH receptor, and both EGF and TGF- $\alpha$  are ligands for the EGF receptor. Even more complexity is evident upon scrutiny of the melanocortin system, in which peptides encoded by different genes, agouti and agouti-related peptide, compete with MSH for binding to melanocortin receptors.<sup>52</sup> Much less common is the biologic paradigm exemplified by OXM and GLP-1, wherein 2 peptides, derived from the same gene,

are ligands for a single common (GLP-1) receptor. Although it remains possible that OXM actions are modified by a separate, as yet unidentified receptor or signaling protein, our data obtained using wild-type and receptor knockout mice convincingly demonstrate that the anorectic actions of OXM require a functional GLP-1 receptor.

Although multiple L-cell-derived peptides secreted from the distal gut modulate food ingestion, the anorectic actions of PYY, GLP-1, and GLP-1R agonists such as exendin-4 have received the most attention because of their demonstrated efficacy in human subjects. Both GLP-1 and exendin-4 bind to the CNS GLP-1 receptor<sup>53</sup> and gain access to the CNS following peripheral administration.<sup>54</sup> The inhibitory effects of GLP-1R agonists on feeding appear mediated through a single known GLP-1R<sup>37</sup> and likely involve specific actions on satiety centers, inhibition of gastric emptying, and modulation of aversive signaling pathways.<sup>12,37,43,55</sup> Indeed, repeated or continuous peripheral administration of GLP-1 or exendin-4 to human subjects produces significant weight



**Figure 6.** <sup>125</sup>I-labeled GLP-1 binds to coronal mouse brain sections. Tissue sections from wild-type (A–D) and GLP-1R<sup>-/-</sup> (E) mice were incubated with 60 pmol/L <sup>125</sup>I-labeled GLP-1 alone (A and E) or in the presence of 200 nmol/L unlabeled GLP-1 (B), oxyntomodulin (OXM; C), or exendin (9-39) (D). CeA, central amygdala; DG, dentate gyrus; HB, habenular nucleus; LH, lateral hypothalamus; VH, ventral hypothalamus; ZI, zona incerta.

loss in clinical studies.<sup>56</sup> In contrast, the potential importance and mechanism of action for the GLP-2–dependent regulation of feeding behavior remains uncertain<sup>14,15</sup> as peripheral infusion of GLP-2 does not modulate appetite or satiety in human subjects.<sup>57</sup>

Although OXM inhibits food intake in fasted or fed rats<sup>22–24</sup> and more recently in human subjects,<sup>25</sup> a separate receptor for OXM has not yet been identified, and the precise receptor and signaling pathway(s) transducing the anorectic effect of OXM remain uncertain. Our data clearly show that, although OXM increases the levels of cAMP in BHK cells through either the rat GLP-1 or glucagon receptors, OXM inhibits food intake exclusively through a GLP-1R–dependent pathway. Nevertheless, our current data, together with studies of OXM and GLP-1 in rats,<sup>24</sup> raise the possibility that OXM and GLP-1R agonists produce differential activation of separate GLP-1R–dependent signaling pathways coupled to inhibition of food intake and energy expenditure.

The sequence of OXM contains all 29 amino acid residues of pancreatic glucagon, followed by an 8 amino acid carboxyterminal extension (Figure 1A); hence, it is not surprising that OXM might mediate its physiologic effects via the glucagon receptor. OXM binds to glucagon receptors and stimulates adenylate cyclase activity in isolated porcine hepatocyte membranes.<sup>29,32</sup> Alternatively, OXM, like glucagon, might interact with both glucagon and GLP-1 receptors.<sup>58</sup> OXM increased cAMP accumulation and stimulated somatostatin secretion through a GLP-1R–dependent mechanism in rat insulinoma-derived RIN T3 cells.<sup>30</sup> Similarly, OXM stimulated cAMP and H<sup>+</sup> production in rat parietal cells in an exendin (9-39)-dependent manner, presumably by interacting with gastric GLP-1 receptors.<sup>31</sup> Although OXM is capable of activating the glucagon receptor in vitro, our studies using knockout mice clearly establish that the anorectic effects of OXM require a functional GLP-1R in vivo.

OXM had no effect on levels of cAMP in BHK-GIPR or BHK-GLP-2R cells, indicating that OXM is unlikely to mediate its actions through these receptors. Nevertheless, our data do not exclude the possibility of OXM-mediated activation of the GIPR or GLP-2R via signaling pathways independent of cAMP, although OXM failed to stimulate inositol phosphate turnover or changes in cellular free Ca<sup>2+</sup> in rat islet cells.<sup>59</sup> OXM interacts with GLP-1 receptors on rat hypothalamic membranes, yet it displays a binding affinity 2 orders of magnitude weaker than that of GLP-1.<sup>22</sup> Similarly, our findings demonstrate that OXM increases cAMP levels with similar efficacy but significantly reduced potency relative to the GLP-1R agonist Ex-4 in BHK-GLP-1R cells, indicating that OXM is a comparatively weak agonist at the GLP-1R.

Our results in wild-type mice suggest that the anorectic effect of ICV OXM is transient, in keeping with a similar response to ICV GLP-1 in rodents.<sup>43,60</sup> In contrast, ICV Ex-4 is more potent and exerts a sustained anorectic effect for at least 24 hours. The greater potency and longer duration of Ex-4 action may reflect its longer half-life because, in part, of its resistance to proteolytic inactivation by the ubiquitous protease dipeptidyl peptidase IV.<sup>61</sup> Although OXM has recently been shown to inhibit food intake in association with reduced circulating levels of preprandial ghrelin in normal human subjects,<sup>25</sup> whether OXM will prove effective in producing weight loss in human patients with obesity remains unknown and requires further investigation.

Does endogenous OXM contribute to regulation of food intake *in vivo*? The GLP-1 receptor is expressed in hypothalamic regions that regulate appetite and feeding behavior.<sup>62,63</sup> PGDPs including OXM are synthesized in the brainstem and, to a lesser extent, in the hypothalamus<sup>64–66</sup> and then transported to distant regions of the CNS. OXM-like immunoreactivity is detectable in the hypothalamus,<sup>67</sup> consistent with an anatomical relationship between OXM, GLP-1R, and hypothalamic centers regulating food intake. Furthermore, IP administration of OXM induced *c-fos* expression in specific brain regions also activated by the GLP-1R agonist Ex-4. Hence, these findings raise the possibility that gut-derived peripheral OXM might also transmit physiologic signals to the brain, consistent with previous findings that peripheral GLP-1 administration activates neuronal *c-fos* in rats.<sup>68</sup> Although our data clearly establish that transient or genetic interruption of GLP-1R-dependent pathways abrogates the anorectic actions of exogenous OXM in mice, whether OXM is physiologically important for control of food intake cannot yet be determined.

ICV OXM elevated core body temperature and caused a disproportionate reduction in body weight compared with pair-fed rats<sup>23,24</sup>; however, our analysis of energy expenditure in mice demonstrated no effect of acute OXM on metabolic rate in wild-type mice. Hence, further experimentation is required to determine whether OXM exerts a species-specific effect on energy expenditure independent of its anorectic actions. In contrast, a single injection of Ex-4, whether given IP or ICV, reduced metabolic rate in mice, consistent with studies examining metabolic effects of GLP-1R agonist administration in rats<sup>69</sup> and with human data demonstrating significantly attenuated diet-induced thermogenesis following a 4-hour intravenous GLP-1 infusion in normal male subjects.<sup>70</sup> Taken together, our data clearly implicate a third member of the PGDP family, OXM, as a

potential regulator of acute nutrient ingestion and provide genetic evidence that a single GLP-1 receptor is capable of integrating signals from related yet structurally distinct intestinal L-cell peptides, which converge on regulation of satiety *in vivo*.

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Address requests for reprints to: Daniel J. Drucker, M.D., Toronto General Hospital, Banting and Best Diabetes Centre, 200 Elizabeth St MBRW4R-402, Toronto, Ontario, Canada M5G 2C4. e-mail: d.drucker@utoronto.ca; fax: (416) 978-4108.

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