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Mu-Opioid Receptors and Dietary Protein Stimulate a Gut-Brain Neural Circuitry Limiting Food Intake

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SUMMARY

Intestinal gluconeogenesis is involved in the control of food intake. We show that mu-opioid receptors (MORs) present in nerves in the portal vein walls respond to peptides to regulate a gut-brain neural circuit that controls intestinal gluconeogenesis and satiety. In vitro, peptides and protein digests behave as MOR antagonists in competition experiments. In vivo, they stimulate MOR-dependent induction of intestinal gluconeogenesis via activation of brain areas receiving inputs from gastrointestinal ascending nerves. MOR-knockout mice do not carry out intestinal gluconeogenesis in response to peptides and are insensitive to the satiety effect induced by protein-enriched diets. Portal infusions of MOR modulators have no effect on food intake in mice deficient for intestinal gluconeogenesis. Thus, the regulation of portal MORs by peptides triggering signals to and from the brain to induce intestinal gluconeogenesis are links in the satiety phenomenon associated with alimentary protein assimilation.

INTRODUCTION

Given the worldwide increase in cases of obesity and associated illnesses such as type 2 diabetes, the scientific community has been urged to improve our understanding of the mechanisms underlying energy homeostasis. An increasingly important area of investigation involves the hormonal signals that the gut produces in response to nutrient assimilation and that modulate hunger, such as cholecystokinin (CCK), glucagon-like peptide 1, and peptide YY (Gibbs et al., 1976; Morley et al., 1983; Turton et al., 1996). In particular, the enteric neural system plays a key role in sensing and transmitting signals to the brain (Berthoud, 2004; Moran, 2000; Vahl et al., 2007).

Intestinal gluconeogenesis is a newly described process (Croset et al., 2001; Mithieux et al., 2004a; Mithieux et al., 2004b; Rajas et al., 1999; Rajas et al., 2000) that influences the control of glucose and energy homeostasis in the fed postabsorptive state (for review see Mithieux, 2009). The induction of intestinal gluconeogenesis translates into a release of glucose in the portal vein, which collects blood from the whole gut. Its detection by a portal glucose sensor and the transmission of this signal to the brain by the peripheral neural system initiate a decrease in hunger and an improvement in the insulin sensitivity of hepatic glucose production. This above all takes place in two particular nutritional situations, i.e., feeding with a protein-enriched diet (PED) (Mithieux et al., 2005; Pillot et al., 2009) and after gastric bypass surgery (Troy et al., 2008). The role played by intestinal gluconeogenesis in the satiety initiated by PED has been confirmed recently. Indeed, mice with an intestinal-specific knockout of the catalytic subunit (G6PC) of glucose-6 phosphatase (G6Pase, the essential enzyme of gluconeogenesis) proved insensitive to PED-induced satiety (Penhoat et al., 2011).

A long-known and intriguing property of proteins is that a number of them, especially those involved in human nutrition such as caseins from milk, release oligopeptides exhibiting μ-opioid activity in vitro upon partial proteolytic digestion (Zioudrou et al., 1979). Moreover, the literature on the subject mentions the μ-opioid activity of various oligopeptides, of which the minimal structure required is a dipeptide (Capasso et al., 1997; Moritoki et al., 1984; Schiller et al., 2002; Shiomi et al., 1981). It is also known that the modulation of μ-opioid receptors (MORs) can interfere with the control of food intake. Agonists enhance food intake, whereas antagonists inhibit it (see the review by Yeomans and Gray, 2002). Interestingly, MORs are most widely expressed in two organs of the body: the brain, especially in the regions implicated in the control of food intake and reward-driven appetite (Ding et al., 1996; George et al., 1994; Will et al., 2003), and the small intestine, where they control gut motility, including bowel movements (Hedner and Cassuto, 1987; Sternini, 2001; Sternini et al., 2004). Food proteins are

absorbed from the intestinal lumen after incomplete proteolysis. It has been hypothesized that food proteins (e.g., caseins) might exert a systemic signaling role via their proteolytic digests with μ-opioid activity (Meisel and FitzGerald, 2000; Zioudrou et al., 1979). However, that the latter might reach the brain following oral ingestion seems questionable. Indeed, β-casomorphin 1-7 (β1-7), an MOR agonist released by the digestion of β-casein, is degraded by the liver (Kreil et al., 1983) and is not detected in systemic blood after the ingestion of milk or dairy products (Teschemacher et al., 1986). Alternatively, MOR modulators from an alimentary origin might act at a gastrointestinal or mesenteric portal site (Holzer, 2009; Meisel and FitzGerald, 2000). In line with this rationale, naloxone (Nalox), a chemical MOR antagonist that is actively degraded by the liver (Greenwood-Van Meerveld et al., 2004; Reimer et al., 2009), can suppress food intake when given orally in humans (Yeomans and Gray, 2002). This suggests that the drug might modulate hunger sensations from a gastrointestinal site.

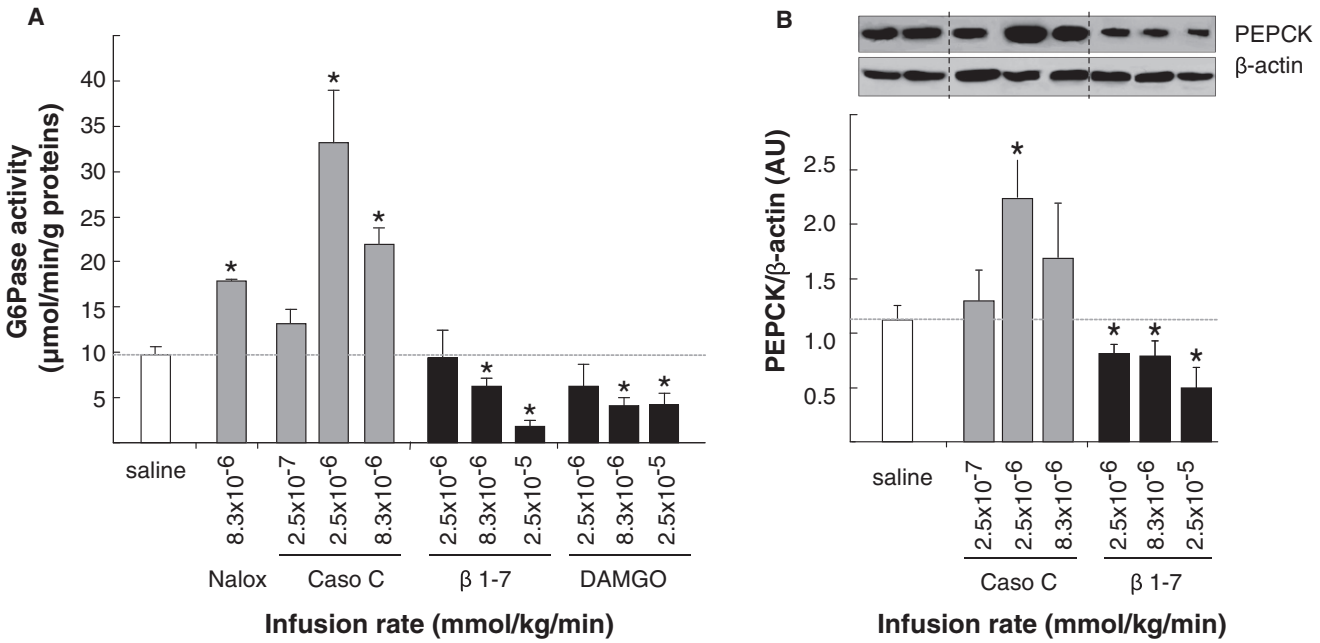
In this work, we first use intraportal infusions of MOR agonists and antagonists to establish that MORs present at a portal site initiate a gut-brain neural circuit of intestinal gluconeogenesis regulation. Then, we demonstrate that alimentary proteins act at this gastrointestinal site to induce gut gluconeogenesis-dependent satiety via the MOR antagonist properties of their oligopeptide digests.

RESULTS

Portal MORs Regulate Intestinal Gluconeogenesis via a Gut-Brain Neural Circuit

To elucidate the role of the portal neural system in the induction of intestinal gluconeogenesis gene expression by food protein, we initially studied the effect of a PED in rats after periportal treatment by capsaicin, a drug that inactivates afferent nerves. Interestingly, there was no induction of either G6Pase activity or PEPCK-C (phosphoenolpyruvate carboxykinase-cytoplasmic form) protein expression in capsaicin-treated rats fed on a PED, unlike what occurred in sham-operated rats in which strong induction was observed (Figures S1A and S1B, available online). We studied the abundance of neuronal fibers, visualized via the presence of the neuronal marker PGP9.5, around the portal vein walls after capsaicin treatment. In agreement with the observation that portal nerves are mainly composed of afferents (Berthoud, 2004), the number of neuronal fibers was dramatically reduced after administration of the drug (Figure S1C). This confirmed the suppression of the afferents by the drug. Then, to test the putative role of MORs in this induction, we infused MOR-regulators in conscious rats with a catheter implanted in a mesenteric vein, as described previously (Mithieux et al., 2005). Three infusion rates were studied. The intermediate rate approximately matched (on a 1:1 molar basis) the appearance of β1-7 from a protein-enriched meal representative of the PED used above. It is noteworthy that both β1-7 and a selective MOR agonist D-Ala2, N3-Me-Phe4, Gly5-ol (DAMGO) significantly suppressed intestinal G6Pase activity after an 8 hr infusion (Figure 1A). On the contrary, both Casoxin C (CasoC), an MOR antagonist stemming from kappa-casein (Chiba et al., 1989) and Nalox markedly enhanced G6Pase activity (Figure 1A). Comparable results were obtained for the expression of the PEPCK-C protein (Figure 1B). When a metabolic glucose tracer (tritiated on carbon 3) was associated with the Nalox infusion at the end of the experiment, there was a decrease in 3[^{3}H] glucose-specific activity in the portal vein compared to that in artery (Figure 1C). This revealed that newly synthesized unlabeled glucose had been released in the blood by the intestine. Intestinal glucose production (IGP) represented 25%–30% of total endogenous glucose production (EGP) after the infusion of Nalox (Figure 1C). IGP was sufficient to counterbalance intestinal glucose uptake (IGU), so no lowering of plasma glucose was observed in the portal vein (Figure 1C). We previously showed that this is sufficient to initiate portal glucose sensing and suppression of food intake (Mithieux et al., 2005; Troy et al., 2008). On the contrary, no such appearance of unlabeled glucose was observed in rats infused with DAMGO (Figure 1C). In this case, there was no decrease in the 3[^{3}H]glucose-specific activity and the level of plasma glucose was lower in the portal vein compared to that in the artery (Figure 1C), reflecting glucose utilization by the gut. It must be noted that we did not determine IGP after infusion of saline. Because of the low accuracy of the tracer dilution method (this is a weakness of this approach), the flux of glucose released by the intestine under basal condition is hardly distinguishable from nil (see e.g., Mithieux et al., 2005; Pillot et al., 2009 for discussion of this point). In agreement with what could be expected from the effect of MOR effectors on intestinal gluconeogenesis, rats infused with MOR antagonist (CasoC) in the portal vein exhibited decreased food intake, whereas those infused with MOR agonist (β1-7) exhibited increased food intake (Figure S1D).

We used three approaches to further determine the role played by portal MORs. Immunohistochemical studies using the neuronal marker PGP9.5 (Figure 2A, in red) revealed the presence of neuronal fibers in the walls of the portal vein both in rats (Figure 2A) and in mice (not shown). In addition, the presence of MOR-1 (green fluorescence) was also detected in close vicinity to the portal vein (Figure 2A). Merging both fluorescence channels (Figure 2A, in yellow) revealed close colocalization of both proteins in certain neuronal structures. Interestingly, we observed comparable colocalization of MOR-1 and PGP9.5 in the portal branches irrigating the portal spaces of the human liver (Figure 2A). Furthermore, the presence of MOR-1 within the portal vein walls was confirmed by western blot (Figure 2B). In contrast, MOR-1 was not detected in the ileum of MOR-KO mice, which confirmed the specificity of the detection (Figure 2B). Then, we studied the effect of β1-7 and CasoC separately on intestinal gluconeogenesis gene expression in capsaicin-treated rats. There was no effect of either effector on gluconeogenic enzymes in rats with portal denervation (Figure S1E), strongly suggesting that the periportal neural system is crucial for the transmission of the MOR-signal. Lastly, we evaluated the impact of β1-7 and Nalox on the regions of the brain implicated in the reception of the signals from the portal area. This is linked to the dorsal vagal complex (DVC), which is the main receiver of inputs from the vagus nerve, and to the parabrachial nucleus (PBN), which mainly receives afferents from the spinal cord (for a review see Berthoud, 2004). Using immunohistochemistry, we studied the expression of the immediate-early gene protein



C

Perfusion	[3-^{3}H]glucose SA (dpm/nmol) Artery	[3-^{3}H]glucose SA (dpm/nmol) Vein	Glucose concentration (mmol/L) Artery	Glucose concentration (mmol/L) Vein	IGU (µmol/kg/min)	IGP (µmol/kg/min)	EGP (µmol/kg/min)
DAMGO	27.5 ± 0.8	27.2 ± 0.9	9.5 ± 0.1	9.1 ± 0.2[a]	8.2 ± 5.0	1.8 ± 1.7	70.4 ± 2.6
Naloxone	29.9 ± 1.4	27.1 ± 1.4[a]	9.2 ± 0.3	9.1 ± 0.3	20.1 ± 4.0	18.1 ± 1.1[b]	65.1 ± 3.0
Tyr-Ala	27.9 ± 1.2	25.1 ± 1.1[a]	8.8 ± 0.6	8.9 ± 0.5	15.2 ± 4.2	18.2 ± 1.7[b]	65.2 ± 2.0
Peptones	31.1 ± 0.5	28.7 ± 0.6[a]	9.4 ± 0.4	9.3 ± 0.3	20.3 ± 2.4	17.4 ± 1.4[b]	69.1 ± 1.1

Figure 1. Effect of Portal Infusion of MOR Effectors on Gut Gluconeogenesis in Rats

(A and B) The rats (fed on an SED) were infused via the portal vein with saline or MOR effectors at the indicated rates, for 8 hr. (A) G6Pase activity. (B) PEPCK-C protein amount estimated by western blot. The data are expressed as the means ± SEM of six to eight rats per group. *, different from saline infusion, $p < 0.05$ (Tukey's post hoc test).

(C) EGP and intestinal glucose fluxes were determined after portal infusions of metabolites (8.3×10^{-6} mmol/kg/min). The results are the means ± SEM of five rats per group. [a]Different from value in artery, $p < 0.05$, Student's two tailed test for paired values. [b]Different from DAMGO, $p < 0.05$, one-way ANOVA followed by Tukey's post hoc test.

See also Figure S1.

product C-FOS, a well-recognized marker of neuronal activation (Bullitt, 1990; Sagar et al., 1988). The portal infusion of β1-7 did not elicit significant C-FOS activation in any of these regions, compared to saline infusion (Figures 3 and S2). On the contrary, Nalox infusion caused a 2- to -3-fold induction of C-FOS in the DVC, i.e., the area postrema (AP) and the nucleus of the tractus solitaris (NTS), and in the PBN (Figures 3 and S2). Interestingly, no activation took place in any of these nuclei in rats whose portal vein had been treated by capsaicin at the time of surgery for catheter implantation (Figures 3 and S2). A very similar pattern of C-FOS activation (and of denervation effect) took place in the main hypothalamic regions, where neurons are projected from the NTS, notably the paraventricular nucleus (PVN), the lateral hypothalamus (LH) and the arcuate nucleus (ARC) (Figure S2). In the same manner, C-FOS activation occurred in the periaqueductal gray (PAG), a nucleus receiving inputs from the PBN, following Nalox infusion, whereas activation did not take place in this nucleus in capsaicin-denervated rats (Figure S2). When surgical disruption of the common hepatic branch of vagus was performed instead of capsaicin denervation, C-FOS activation induced by Nalox was cancelled in the NTS and in the downstream PVN but was still present in the PBN and in the PAG (Figure S3).

Proteolytic Digests and Oligopeptides Exhibit MOR Antagonist Properties

When rats were previously fed on a PED for 3 days, there was a marked increase in amounts of G6PC and PEPCK-C protein

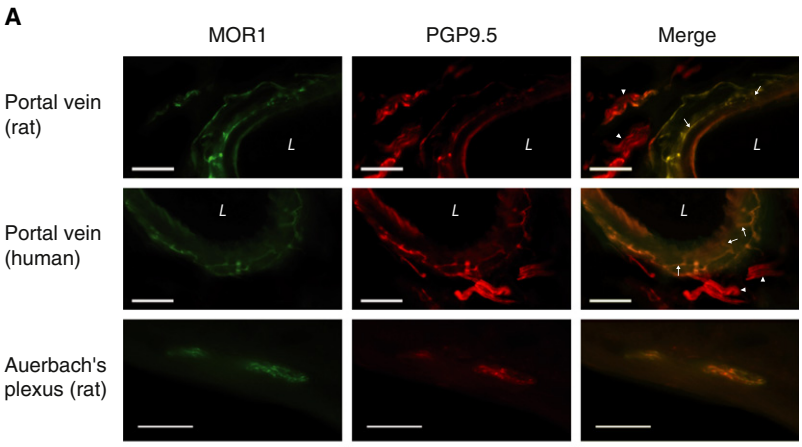


Figure 2. Presence of MORs within the Walls of the Portal Veins in Rats and Humans

(A) MORs (green, left) colocalized with specific neuronal marker ubiquitin carboxy-terminal hydrolase PGP9.5 (red, middle) in the walls of the portal vein of both rats and humans. Right: merged images. The arrows point out the neural fibers innervating the wall of the portal vein. The arrowheads point out nerve bundles in the vicinity of the portal vein. Bottom:, MOR was visualized in the Auerbach's plexus (the nervous system innervating the muscular layers of the intestinal wall, which controls motility) as a positive control. White bar: 50 μm in portal veins, 25 μm in the Auerbach's plexus. The data shown are representative of portal veins from three rats and portal branches from four different human samples.

(B) Western blot analysis of the presence of MOR-1 in the ileum and the portal vein of rats and its absence in the ileum of MOR-KO mice as a control of specificity.

(compare Figure 4 and Figures 1A and 1B). Infusing MOR agonists in the portal vein of PED-fed rats reversed increases of G6PC and PEPCK-C protein. On the contrary, no additional induction effect took place when infusing MOR antagonists (Figure 4). The same pattern was observed with a PED containing a mixture of milk caseins (which might possibly release β1-7 or CasoC into the blood) (Figure 4), or with a PED from plant proteins (i.e., soy, which did not contain these moieties) (Figure S4). This suggests that MOR antagonists and PED from any origin could induce intestinal gluconeogenesis gene expression via the same mechanistic pathway.

To document the above assumption, we combined several approaches. First, we infused a proteolytic digest (peptones) and selected oligopeptides (di- or tripeptides) separately into the mesenteric-portal vein of rats. The following structures were selected to match the di- or tripeptides with or without a lateral chain (Tyr-Ala and Gly-Gly, respectively) or with an electric charge in the lateral chain (Phe-Pro-Arg). In all cases, a marked induction of G6Pase was observed in both the jejunum (Figure 5A) and the ileum, a gut portion of weaker G6Pase activity in the basal state, but inducible in the case of PED feeding (Mithieux et al., 2005) (Figure 5B). In Figure 5, peptones were from a mixture of caseins. However, peptones from meat protein had the same effect (not shown). In addition, glucose tracer dilution studies revealed that IGP took place after the portal infusion of Tyr-Ala and peptones separately (Figure 1C). Finally, as observed previously for MOR antagonists (Figure S1), no induction of G6Pase by oligopeptides took place in rats with capsaicin denervation of the periportal area (Figure 5). Second, in rats infused with Tyr-Ala, there was a 3-fold increase in C-FOS expression in the DVC (Figures 3B and 3G) and in the hypothalamus, the PBN and the PAG (Figure S2), just like the rats infused with Nalox. Furthermore, no increase in C-FOS took place after inactivation of portal afferents (Figures 3B, 3H, and S2). Third, we tested whether or not oligopeptides could behave as MOR antagonists in a neuroblastoma cell constitutively expressing MOR (Lorentz et al., 1988; Yang et al., 2000). (1) We checked that labeled (tritiated) DAMGO bound to N1E-115 cells and that unlabeled DAMGO competed for binding with a 50% inhibitory concentration (IC_{50}) of about 10^{-7}M, thus confirming previous results (Zhang et al., 2006). Then, we showed that Tyr-Ala and Gly-Gly competed for the binding of tritiated DAMGO with IC_{50} means of 3.10^{-6}M and 9.10^{-7}M, respectively. Peptones also competed efficiently with tritiated DAMGO (data not shown). (2) We studied the effect of oligopeptides on the coupling of MOR to adenylate cyclase in the same cells. Concomitant with the fact that MOR is coupled via G-protein-inhibiting adenylate cyclase, the agonist DAMGO strongly decreased cell cAMP content (Figure S5). On the contrary, the antagonists CasoC and Nalox dramatically increased cell cAMP content. This is in keeping with a previous observation: an endogenous activity of MOR may exist, constantly depressing the cAMP level even in the absence of an agonist. This endogenous activity may be eliminated on antagonist binding, thus promoting an increase of cAMP (Kotz et al., 2000). It is noteworthy that all oligopeptides increased the cAMP content and could prevent the decrease of DAMGO-induced cAMP in coincubation experiments (Figure S5). This strongly suggests that oligopeptides exhibited MOR antagonist properties.

MOR-Dependent Induction of Intestinal Gluconeogenesis Is a Causal Link in the Satiety Effects of Food Proteins

To demonstrate the causal role of MORs in the protein-digest-dependent induction of intestinal gluconeogenesis and its role in associated satiety, we performed experiments of portal infusion on mice with deletion of *Mor* (MOR-knockout) and on mice with intestine-specific deletion of G6Pase (I-*G6pc*-knockout, G6PC being the catalytic subunit of G6Pase). There was a slight basal induction of gluconeogenic enzymes in

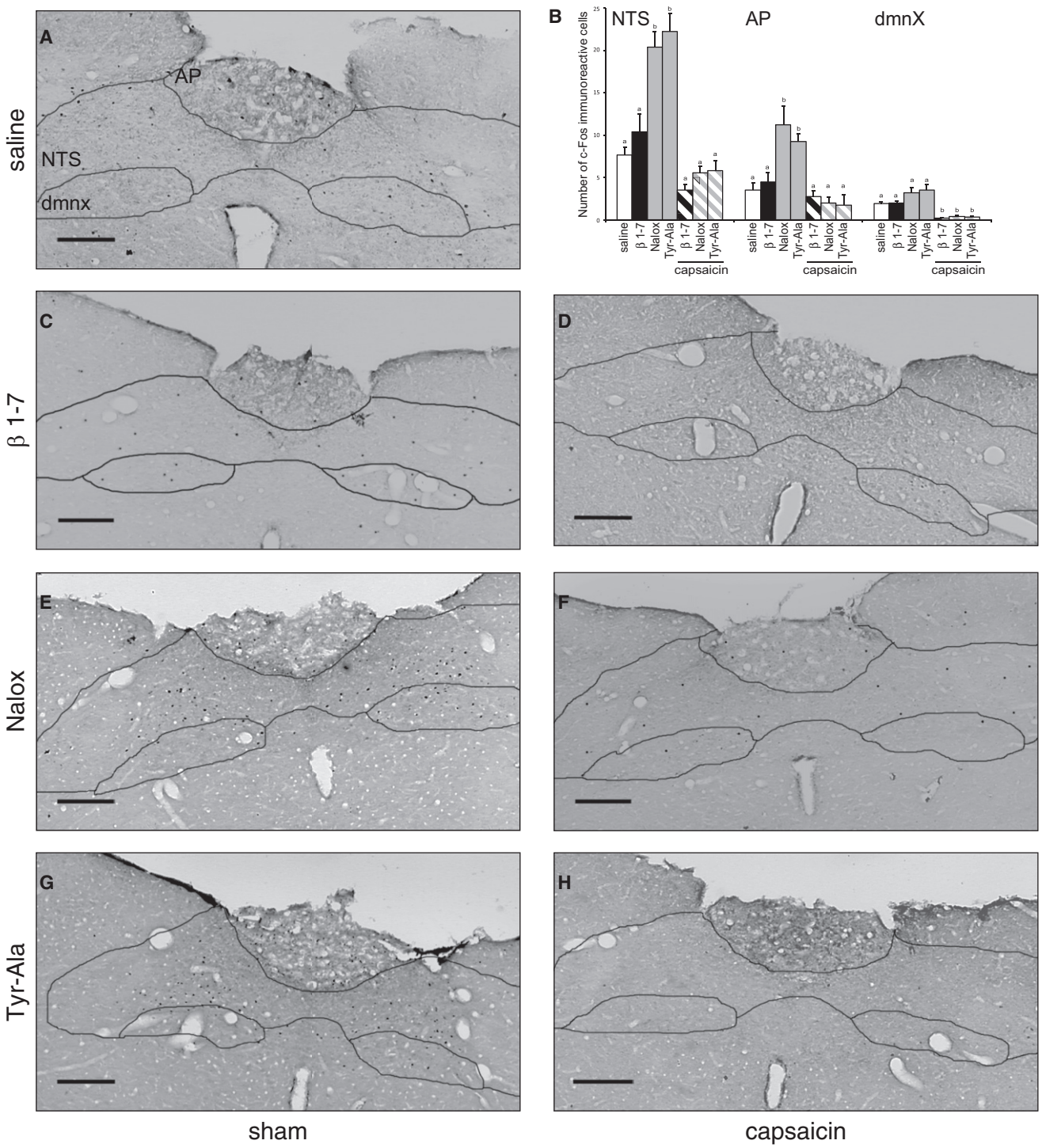


Figure 3. Effect of Portal Infusion of MOR Agonists and Antagonists on C-FOS Expression in the Dorsal Vagal Complex of Rats

(A, C, E and G) C-FOS immunoreactive cells in the DVC of rats infused via the portal vein with saline only (A), β1-7 (C), Nalox (E), and Tyr-Ala (G) (at a rate of $8.3.10^{-6}$ mmol/kg/min).

(B) Quantification of C-FOS neurons in all areas of the DVC: saline (white bar), β1-7 (black bar), Nalox (gray bar). Open bars: sham-treated rats; dashed bars: capsaicin-treated rats. Data are expressed as means ± SEM (n = 3) of immunoreactive cells per brain hemisphere. The values with different letters differed significantly ($p < 0.05$, Kruskal-Wallis nonparametric test, followed by Dunn's post hoc test).

(D, F, and H) Comparable infusions of β1-7 (D), Nalox (F) and Tyr-Ala (H) in rats previously treated by capsaicin around the portal vein.

Scale bar: 200 μm. The following abbreviations are used: AP, area postrema; NTS, nucleus of the solitary tract; dmnX, dorsal motor nucleus of the vagus. See also Figures S2 and S3.

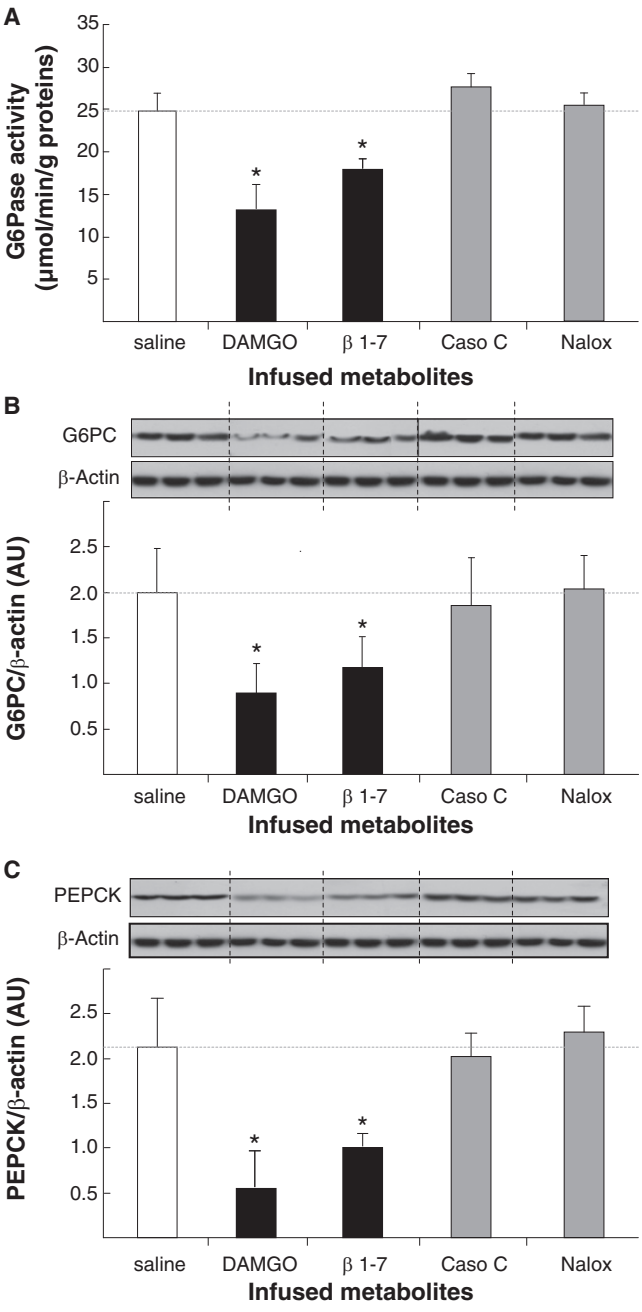


Figure 4. Effect of Portal Infusions of MOR Effectors on Gut Gluconeogenesis Gene Expression in Rats Fed with PED

Rats with portal catheters were fed the PED diet for 2 weeks before infusion. Infusion with MOR effectors was performed as in Figure 1.

(A) G6Pase activity.

(B and C) Quantification of G6PC and PEPCK-C proteins by western blotting. The data are expressed as the means ± SEM (n = 5). *, value significantly different from the saline infusion group, $p < 0.05$.

See also Figure S4.

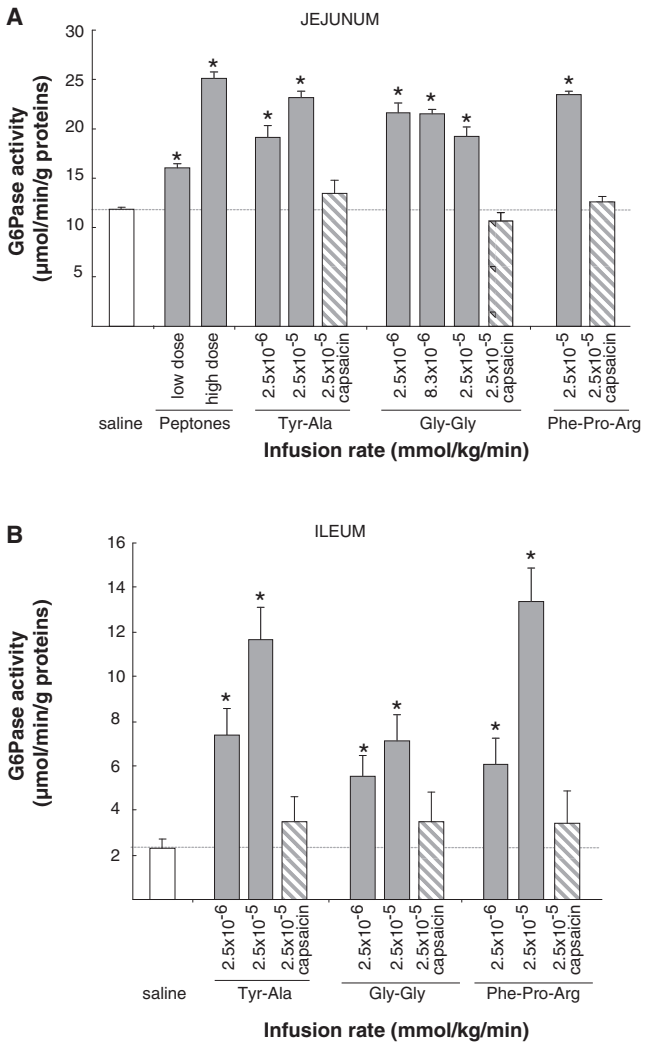


Figure 5. Effect of Oligopeptides and Protein Digests on G6Pase Enzyme Expression in the Rat Intestine

Rats with portal catheters (fed on SED) were infused for 8 hr with the indicated metabolites at the indicated rates. The rate of infusion of peptones was calculated to match the rate of appearance of protein digests derived from the assimilation of an SED (low dose) or PED (high dose). The shaded bars refer to experiments in rats treated locally with capsaicin around the portal vein.

(A) Glc6Pase activity in jejunum.

(B) Comparable determinations performed in a distal ileum segment. The data are the means ± SEM (n = 6). Statistics are as described in the legend of Figure 1.

See also Figure S5.

MOR-KO mice (Figures S6A and S6B). Again, this is in line with the basal tone of MORs in wild-type (WT) mice (see above), which would not exist in MOR-KO mice. When infused in WT mice, di- and tripeptides and peptones induced G6Pase activity in the jejunum (Figure 6A). On the contrary, DAMGO suppressed G6Pase activity, and this was inversed by peptones in coinfusion experiments (Figure 6A). It is noteworthy that none of these effectors induced any effect on gut gluconeogenesis in MOR-KO mice (Figure 6B). Furthermore, we studied food intake in MOR-KO and WT mice after a switch from starch-enriched diet (SED) to PED. Food intake per day for both groups was not different with SED. After a transient drop, a classic response to

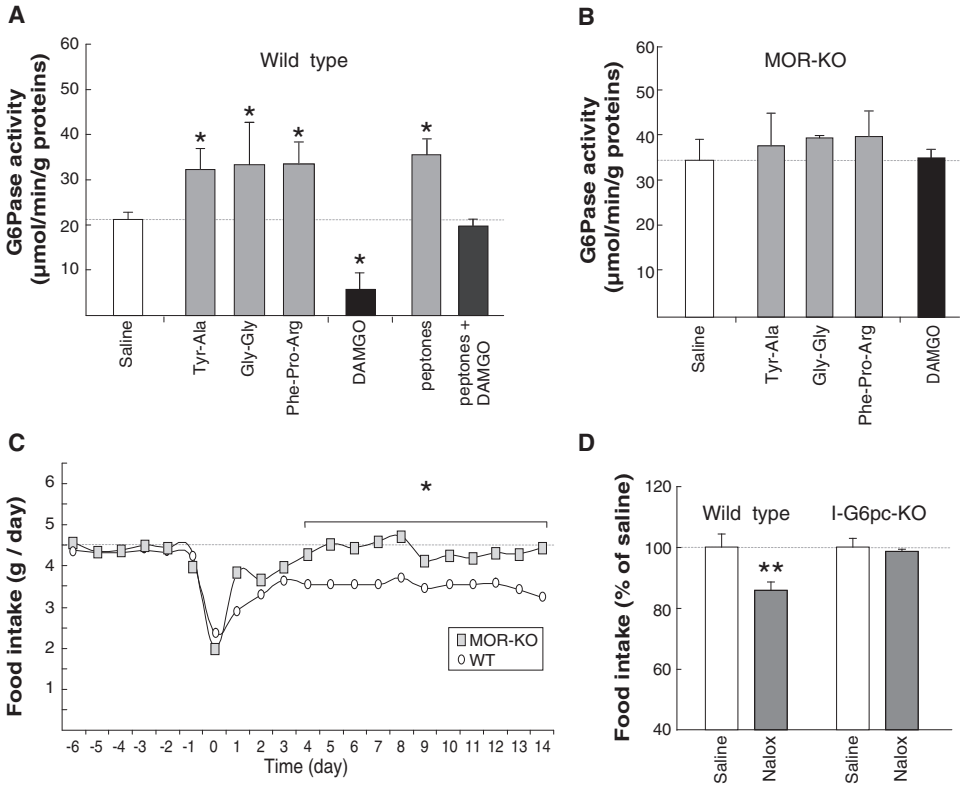


Figure 6. Effect of MOR Modulators and Oligopeptide Fractions on Intestinal G6Pase Enzyme Expression and Features of Food Intake in Wild-Type and MOR-KO or I-G6pc-KO Mice

(A) G6Pase activity was determined in WT mice (fed on SED) with a portal catheter, which were infused with the indicated metabolites at a rate of 8.3 × 10^{-6} mmol/kg/min for 8 hr. The expression of the data and statistical analysis are as described in the legend of Figure 1 (n = 6 mice per group).

(B) Comparable experiments performed on MOR-KO mice.

(C) Food intake was monitored each day during the first period of feeding SED to WT (open circles) and MOR-KO (gray squares) mice. At day 0, the mice were switched to PED and food intake was monitored for a further 2 weeks. The data are expressed as the means ± SEM of eight mice per group. The error bars are not shown for the sake of graph clarity. All values on day 4 were different between the WT and MOR-KO mice ($p < 0.05$, Student's t test for unpaired values).

(D) Saline or Nalox (8.3 × 10^{-6} mmol/kg/min) were infused alternatively in WT and I-G6pc-KO mice for 24h. The amount of food ingested was quantified at the end of the infusion. The data are expressed as mean ± SEM (WT: n = 6; I-G6pc-KO: n = 6). **, different from saline, $p < 0.01$ (Student's two tailed test for paired values). See also Figure S6.

changes in food type in rodents, food intake was reduced by about 20% in WT mice fed with a PED. On the contrary, MOR-KO mice resumed their previous food intake (Figure 6C). It is noteworthy that this insensitivity to PED was linked to an absence of modulation of intestinal G6Pase. This highlights a dramatic difference from the induction effect shown in WT mice (Figure S6C). Lastly, we studied the food intake in WT and I-G6pc-KO mice infused with an MOR antagonist via the portal vein. There was a 15% decrease in the amount of food ingested in WT mice infused with Nalox (Figure 6D), i.e., mice exhibiting increased intestinal gluconeogenesis (Figure 6A). In contrast, no effect was observed upon the infusion of Nalox in I-G6pc-KO mice, i.e., in the absence of intestinal gluconeogenesis (Figure 6D). Comparable results were obtained when Tyr-Ala was infused instead of Nalox (13% decrease in the amount of food ingested in WT mice, no diminution in I-G6pc-KO mice). Taken together, these data strongly suggest that MOR-mediated intestinal gluconeogenesis and MOR-mediated modulation of hunger are closely linked phenomena, which may account for the satiety effect of food protein following their assimilation.

DISCUSSION

By combining infusions of MOR effectors in the portal vein in conscious rats and experiments of denervation of the portal vein walls, we demonstrate that MORs associated with portal neuronal fibers initiate a gut-brain neural circuit of induction of intestinal gluconeogenesis, a function controlling food intake (Mithieux et al., 2005). An infusion into the gut lumen would better mimick the in vivo appearance of protein digests. However, the flux of native peptide metabolites is difficult to monitor from the gut lumen due to its high protease content. The neural system immediately surrounding the intestinal mucosa is by-passed under the experimental conditions of portal infusion. Therefore, even if infused metabolites are sensed in the portal area under the experimental conditions studied here, it cannot be excluded that MOR-dependent sensing may also, under

physiological conditions, take place upstream in the neural system surrounding the gut mucosa or in the gut muscular layer (Hedner and Cassuto, 1987; Sternini, 2001).

Neural afferents traveling along the ventral trunk of the vagus nerve are likely to be involved in the transmission of the MOR signal to the brain. In support of this view, the activation of both the NTS and hypothalamus induced by MOR antagonists (revealed by C-FOS expression) is suppressed either by capsaicin treatment or by surgical vagotomy. However, the signals conveyed via the spinal cord, i.e., spino-mesencephalic route (PBN and PAG), are likely to be involved as well. In agreement with this assumption, both the PBN and the PAG, which connect afferents from the spinal cord, are activated via portal MOR antagonists. Moreover, the activation in these nuclei is suppressed via periportal treatment by capsaicin (a treatment that ablates the afferents of either route) and not by surgical vagotomy (a treatment that preserves the spinal afferents). Interestingly, the NTS, hypothalamus, and spino-mesencephalic pathways are all known to be involved in the control of energy homeostasis (see Berthoud, 2004 for a review).

It is established that proteins are partially digested within the gut lumen to oligopeptide moieties mainly composed of di- or tripeptides (Daniel, 2004). These oligopeptides are then transported within enterocytes by means of a specific transporter (PepT1) of the luminal membrane (Boll et al., 1994; Nielsen and Brodin, 2003). A fraction undergoes further proteolysis to amino acids to feed the intestinal amino acid pool. Another fraction is released into the portal blood, together with amino acids, via specific oligopeptide and amino acid transporters located at the basolateral membrane (Lee, 2000). A number of proteins have been shown to release proteolytic digests with μ-opioid activity (see above). However, the assumption that any food protein could induce intestinal gluconeogenesis via a MOR-dependent pathway implied that the proteolytic digests or peptides of any protein origin could exhibit MOR antagonist activity. This inference is strongly supported by the observation herein that peptides, either alone or in mixture (peptones), behave as MOR antagonists both in vivo to induce gut gluconeogenesis and in vitro in experiments of coupling to adenylate cyclase. The absence of these regulations in MOR-KO mice and their insensitivity to PED feeding, along with the incapacity of MOR antagonists to modulate food intake in mice with ablated intestinal gluconeogenesis, together confirm a causal link between MOR antagonism by oligopeptides and the satiety effect induced by food protein.

These findings may appear difficult to reconcile with the literature reporting that oligopeptides and food protein digests could behave as either MOR agonists or MOR antagonists (Capasso et al., 1997; Moritoki et al., 1984; Schiller et al., 2002; Shiomi et al., 1981, Zioudrou et al., 1979). However, following ligand-receptor interaction, various MOR-initiated cellular processes occur, such as receptor internalization, intracellular sorting and recycling (Böhm et al., 1997; Sternini et al., 2000). This results in complex adaptations including receptor downregulation, desensitization, and resensitization, depending on the concentration of the ligand and on the time of exposure. These cumulative phenomena make the resulting downstream effects of MOR-modulators (i.e., agonist-like or antagonist-like) almost unpredictable (for a review see Taylor and Fleming, 2001). Altogether, this probably accounts for some of the inconsistent results published in the field and may also explain, at least partially, why the different metabolites studied herein did not always elicit clear dose-dependent effects (see e.g., Figure 1 or Figure 5). In addition, the *Mor* gene family is highly complex and includes multiple splice variants and various promoters, depending on its expression site (Pan, 2005). These splice variants may be functionally distinct, regarding their regulation by various agonists or antagonists, or depending on their location (Pan, 2005; Pasternak et al., 2004). Therefore it is possible that the general antagonist behavior of peptides and protein hydrolysates revealed here is a specific feature of MORs expressed in the mesenteric-portal system.

Another intriguing question relates to the complexity of the sequence of events, which allows the alimentary protein to exert their satiety end-point action. Indeed, a number of known factors, e.g., intestinal hormones, arise from the gut driven by the meal and are assumed to affect food intake by binding their receptors directly in the hypothalamus. These are rapidly acting mechanisms. In the case of food proteins, a series of events initiates the gluconeogenesis gene program in the intestine, via a reflex arc with the brain. This is progressive and takes place over the postprandial period (Figure 7). After that, the glucose released may initiate its central action via portal-sensing driven mechanisms (Mithieux et al., 2005). This is a lasting phenomenon because it depends on gene induction and thus may endure after food assimilation. This is in line with the fact that protein feeding decreases hunger some time from the preceding meal, which is the definition of the phenomenon called ''satiety'' (Booth et al., 1970; Rolls et al., 1988). This might explain why protein feeding decreases food intake in the long term, i.e., for several days or weeks (Jean et al., 2001; Mithieux et al., 2005; Penhoat et al., 2011). Hence, advantages exist in terms of reduction of body weight and adipose tissue mass (Jean et al., 2001, Mithieux et al., 2005). On the contrary, rodents treated before meals with the satiation hormone CCK, which is involved in the meal termination, do not reduce their global food intake. Indeed, they compensate the CCK-initiated decrease of meal size by increasing the number of meals per day (West et al., 1984). To evaluate the long-term potential effect of MOR modulators, lasting portal infusion experiments would have been of interest. It is likely that benefits on the adipose tissue mass and body weight would be similar to those observed with protein feeding. Unfortunately, technical problems (portal catheters blocked or pulled out) are very frequent after a few days, making such experiments infeasible.

In conclusion, we report that MORs expressed in the mesenteric-portal area control a gut-brain neural circuit of regulation of intestinal gluconeogenesis. The latter controls food intake (Mithieux et al., 2005; Penhoat et al., 2011, Troy et al., 2008). The regulatory role of MORs in the control of food intake has been largely documented for the central nervous system, related to their roles in the so-called ''reward'' system (Glass et al., 1999; Shin et al., 2010). Here, we report that MORs play a role in mediating the satiety effects of alimentary proteins, acting within a neural gut-brain circuit (Figure 7). Remarkably, various μ-opioid antagonists given orally decrease hunger in humans (Yeomans

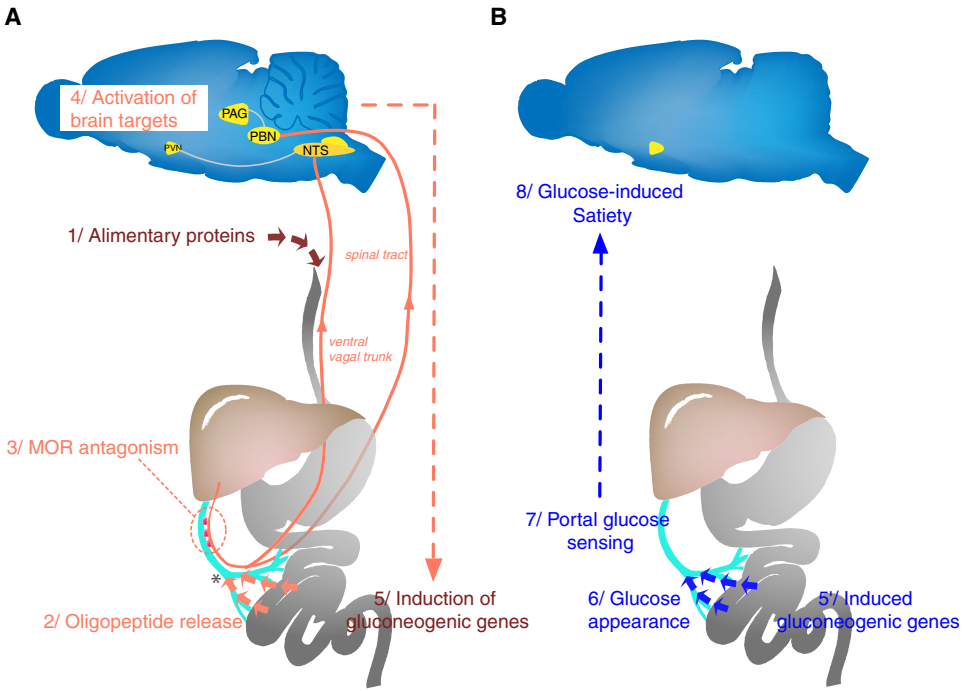


Figure 7. Sequence of MOR-Dependent Induction of Satiety by Food Protein

(A) During the postprandial period, the oligopeptides resulting from proteolysis of alimentary proteins are released in the portal blood and antagonize MORs expressed in the periportal neural system. The ascending nerves controlled by MORs, traveling via both the vagus nerve and the spinal cord, convey the information to their respective brain targets, driving the induction of intestinal gluconeogenesis gene expression. *, refers to the site of appearance of the metabolites infused in this study.

(B) During the postabsorptive period, glucose released in the portal blood is sensed by the portal glucose sensors and exerts its satiety effects (Mithieux et al., 2005).

and Gray, 2002), despite the fact that they do not reach the brain due to extensive first-pass hepatic metabolism (Reimer et al., 2009). Gastric bypass, an increasingly popular surgical operation treating morbid obesity, promotes a dramatic reduction of hunger sensation in a few days. This is true in obese mice (Troy et al., 2008) and in obese humans (for a review see Thaler and Cummings, 2009). Although intestinal gluconeogenesis was revealed to be involved in the decreased food intake after gastric bypass in mice (Troy et al., 2008), a net portal release of glucose under postabsorptive conditions has been observed several days after gastric bypass in nondiabetic obese patients (Hayes et al., 2011). This suggests that intestinal gluconeogenesis could be sufficient to compensate and even surpass gut glucose utilization. As MOR-positive neural fibers are present in the wall of the human portal vein branches (Figure 2A), the mechanisms uncovered here may also take place in humans. This knowledge may thus pave the way for future approaches in the treatment and/or prevention of metabolic diseases.

EXPERIMENTAL PROCEDURES

Animals and Tissue Sampling

Adult male Sprague-Dawley rats (Charles River), aged 6 to 8 weeks and weighing 260–280 g at the time of their arrival, were housed in a climate-controlled room (22 ± 2°C) subjected to a 12 hr light/dark cycle with lights on at 7:00 AM and lights off at 7:00 PM, with free access to water and an SED diet. Mice were housed under similar conditions. A colony of MOR-KO mice (Jackson Laboratories) was generated in our facilities from two couples of mice. I-G6pc-KO mice were described previously (Penhoat et al., 2011). The experiments were performed 5 weeks after gene deletion. PED composition was based on SED (SAFE A04, France), with a modification of starch-glucose: protein ratio from 50:23 to 23:50 (weight basis). Proteins were an equimolar ratio of soy proteins and casein. However, soy proteins were used for only a few experiments. The intestine was sampled as described previously (Mithieux et al., 2004a). Enzyme expression analyses were performed in proximal jejunum in all but a few experiments (Figure 5B). Glc6Pase activity was assayed under conditions of maximal velocity. All procedures were described in detail previously (Mithieux et al., 2004a; Rajas et al., 1999).

Studies in Conscious Animals

Animals were anesthetized with 2% isoflurane. A catheter was implanted in the inferior left mesenteric vein after laparotomy and secured with biological glue. A passage under the skin up to the neck was done to recover the other end of the catheter, which was secured with a Dacron mesh button tether (Harvard apparatus). The procedure to implant a portal catheter (PE10, Fine-Bore Polyethylene Tubing, Smiths Medical) in WT and I-G6pc-KO mice was comparable. In rats intended for portal vein denervation, a gauze compress moistened with 80 μl of a capsaicin solution (10 mg/ml) dissolved in water, ethanol, and Tween at a ratio of 8:1:1 was applied around the portal vein for 15 min. Regarding vagotomy studies, the left and caudal lobes of the liver were gently deflected, and the hepatic branch of the vagus, along with the fascia surrounding the nerve, was sectioned with fine scissors. Animals were allowed to recover from the surgery for 1 week, with free access to SED and water. A marbocyl/ketofen solution was infused each day, in order to prevent coagulation, infection, and pain.

Portal infusions (at a rate of 125 μl/hr for rats and 15 μl/hr for mice) were carried out for 8 hr, starting at 9 A.M., in animals with free access to food and water. Saline was infused as a control vehicle. The following metabolites were infused: [D-Ala_2, N_3-Me-Phe_4, Gly_5-ol]-enkephalin (DAMGO), human β1-7, Nalox, peptones from meat protein (Sigma-Aldrich), CasoC (kindly provided by Damien Ficheux, IBCP, Lyon, France), dipeptides H-Tyr-Ala-OH and H-Gly-Gly-OH, and tri-peptide H-Phe-Pro-Arg-OH (Bachem). Following the infusions, the animals were anesthetized with sodium pentobarbital for tissue sampling.

To study food intake in WT and MOR-KO mice (Figure 6C), basal consumption was determined for 5 days before switching to PED. Then, we measured food intake every day for 15 days. To study the effect of Tyr-Ala in WT and l-G6pc-KO mice (Figure 6E), portal infusions were carried out for 24 hr and the amount of food ingested quantified at the end. Each mouse was studied alternatively for saline or Nalox infusion, including 1 day without experiment between infusions.

Determination of Intestinal Glucose Fluxes

After an 8 hr infusion of metabolites, as described above (but with no food for the last 6 hr), rats were anesthetized and fitted with polyethylene catheters inserted into the right jugular vein for 3[^{3}H]glucose infusion and into the left carotid artery for blood sampling, as described previously (Croset et al., 2001; Mithieux et al., 2005; Rajas et al., 1999). At the end of the additional infusion time (for 90 min), blood was sampled from the carotid artery and the portal vein. Blood plasma was separated and deproteinized and used to determine glucose concentration and specific activity (SA). All calculations were done by using the formulas described in detail by Croset et al. (2001).

Microscopy Studies

Animals were deeply anesthetized with intraperitoneal injection of sodium pentobarbital, and transcardially perfused with 0.9% NaCl followed by ice-cold phosphate-buffered (pH 7.4) paraformaldehyde (PFA). Tissues were postfixed in PFA for 2–3 hr and cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) with thimerozal (0.01% v/v). Before freezing, the samples were embedded in Tissue-Tek (Sakuro Finetek), and vessels and portions of the intestine were injected with the same substance. Human portal branches were sampled from healthy portions of hepatectomized livers and immediately postfixed by immersion in PFA as described above. Serial 20 μm thick sections were cut on a freezing cryostat.

Immunofluorescence

Sections were preincubated in PBS with 0.3% Triton X-100 (PBST) containing 5% bovine serum albumin (blocking solution) for 2 hr and further incubated with the primary antibody in the blocking solution overnight at room temperature. After several rinses in PBST, sections were incubated in the secondary antibody for 2 hr at room temperature. For double labeling, the protocol was repeated with the other antibodies after three 15 min washes in PBST. Staining was absent in the control experiment with omission of primary antibodies. Furthermore, we checked that no cross-reactivity between antibodies occurred, as assessed by inverting the order of staining and omitting the second primary antibody (data not shown). Images were generated by using a fluorescence microscope (Carl Zeiss Axiovert 200M).

See also Table S1.

C-FOS Labeling

Prior to any incubation, endogenous tissue peroxidase activities were quenched with 1% H_2O_2 in PBS for 30 min. The procedure was then the same as described before for primary and secondary antibody incubations. Following PBS washes, the ABC Elite reagent (Vector Laboratories) was applied for 45 min. The sections were then incubated in 3,3′-diaminobenzidine (DAB), in conjunction with 5 mg/ml nickel and ammonium sulfate (Sigma-Aldrich) for 15 to 30 min at a concentration of 0.5 mg/ml in PBS with 0.03% H_2O_2. Sections were thoroughly washed, air-dried, dehydrated, and coverslipped. For the quantitative assessment of C-FOS expression in the brain, four to six sections were chosen in areas in the hypothalamus, PBN, and PAG, as well as in the brain stem, according to Paxinos and Watson (1998). Images were generated with a light microscope (Nikon Eclipse E400) and FOS-positive nuclei were counted by using a calibrated macro as described before (Zheng et al., 2002) with ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). All data are expressed as FOS-positive neurons per hemisphere of the brain.

See also Table S1.

Statistical Analyses

Data are expressed as means ± SEM. One-way ANOVA followed by Tukey's post hoc test was used for multiple parametric comparisons. Student's two-tailed paired t test was used for paired comparisons. The nonparametric Kruskal-Wallis test followed by Dunn's post hoc test was used for C-FOS positive neuron counting. Statistical significance was set at $p \leq 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.05.039.

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