

SUPPLEMENTAL DATA FILE

Figure S1 (related to Figure 1): Effect of PED or of MOR-modulators on intestinal gluconeogenesis enzymes and food intake in normal and capsaicin-treated rats:

A, B) Rats were treated by capsaicin around the portal vein or sham-operated. After recovery for 1 week fed on SED, they were switched on PED for 3 days and studied in comparison with a sham-operated group fed on SED. G6Pase activity (**A**) and PEPCK-c protein (**B**) were quantified in jejunum. The data are expressed as the means \pm SEM of three rats per group. *, significantly different from SED-sham, $p < 0.05$.

C) Disappearance of nerve fibers in the walls of portal vein after capsaicin treatment. Immunofluorescence labeling of nerve fibers (neuronal marker PGP9.5) of portal vein was carried out in rats after sham (left panel) or capsaicin (right panel) treatment. Capsaicin effectively abolished the neural marker PGP9.5 immunoreactivity. L=luminal part of the vein. The scale bar represents 200 μ m.

D) Rats with portal catheters were infused for two days with the indicated metabolites as in panel D and the food intake was monitored at the end of the first and second days. The data are expressed as the means \pm SEM of six to eight rats per group. *, significantly different from saline infusion, $p < 0.05$.

E) Rats underwent surgery for a portal catheter implantation. In rats aimed at portal vein neural inactivation, capsaicin treatment was performed at the time of surgery. After one week of recovery on SED, they were infused with the indicated metabolites for 8h at a rate of 8.3×10^{-6} mmol/kg/min. G6Pase activity was quantified as above. The data are expressed as the means \pm SEM of five rats per group. *, Significantly different from saline infusion, $p < 0.05$.

Figure S2 (related to Figure 3): Effects of the portal infusion of MOR modulators on c-Fos expression in the PBN/ PAG and hypothalamus of rats:

A, C, E and G) c-Fos immunoreactive cells in the PBN of rats infused via the portal vein with saline only (**A**), β 1-7 (**C**), Nalox (**E**) and Tyr-Ala (**G**). (Rate of infusion: 8.3×10^{-6} mmol/kg/min).

D, F, H) Comparable infusions of β 1-7 (**D**), Nalox (**F**) and Tyr-Ala (**H**) in rats previously treated by capsaicin around the portal vein. The scale bar represents 200 μ m.

B) Quantification of c-Fos neurons in the PBN and PAG: saline (white bar), β 1-7 (black bar), Nalox (grey bar). Open bars: infusions in sham-treated rats; dashed bars: infusions in capsaicin-treated rats. Data are expressed as means \pm SEM of immunoreactive cells per brain hemisphere ($n = 3$ per experimental group). The values with different letters differed significantly ($p < 0.05$, Kruskal and Wallis non-parametric test, followed by Dunn's post-hoc test). PBN: parabrachial nucleus, PAG: periaqueductal grey.

I, K, M, O) c-Fos immunoreactive cells in the PVN of rats infused via the portal vein with saline only (**I**), β 1-7 (**K**), Nalox (**M**) and Tyr-Ala (**O**).

L, N, P) Comparable infusions of β 1-7 (**L**), Nalox (**N**) and Tyr-Ala (**P**) in rats treated by capsaicin. The scale bar represents 100 μ m.

J) Quantification of c-Fos neurons in the LH, ARC and PVN: symbols, expression of data and statistics as above. LH: lateral hypothalamus, ARC: arcuate nucleus, PVN: paraventricular nucleus.

Figure S3 (related to Figure 3): Effect of portal infusion of Nalox on c-Fos expression in rats with surgical vagotomy:

A – D) Rats underwent a surgical vagotomy. After recovery, they were infused with saline (left panel) or Nalox (right panel). c-Fos immunoreactive cells were quantified in the NTS (**A, B**) and in the PBN (**C, D**). Infusion rates were as in Fig.3. Scale bars represent 200 μ m.

E) Quantification of c-Fos positive cells in the NTS, PVN, PAG and PBN: saline (white bar) and Nalox (grey bar). Data are expressed as means \pm SEM (n=3) of immunoreactive cells per brain hemisphere. *, p <0.05, using Mann-Whitney's non-parametric test.

Figure S4 (related to Figure 4): Effect of portal infusions of MOR modulators on gut gluconeogenesis enzyme expression in rats fed on a PED from soy protein:

A) Rats with portal catheters were fed on a PED from soy protein for two weeks before infusions. Infusions with MOR-effectors were done as described in Figure 4. G6Pase enzyme activity was determined in a jejunum sample.

B) Quantification of PEPCK-C protein by western blotting. The data are expressed as the means \pm SEM of five rats per group. *, Significantly different from saline infusion, p < 0.05.

Figure S5 (related to Figures 5): Effect of MOR modulators and oligopeptides on the coupling of MOR to adenylate cyclase in N1E115 cells:

N1E115 cells were incubated for 20 min at 37°C in the presence of the indicated metabolites (10^{-7} M). cAMP was assayed by radioimmunoassay using 125 I-labelled cAMP. The effect of forskolin was studied as a positive control. The data are expressed as the means \pm SEM of four incubation experiments. *, Significantly different from saline; °, Significantly different from DAMGO alone; p < 0.05.

Figure S6 (related to Figure 6): Determination of gluconeogenic enzyme expression in wild-type and MOR-KO mice:

A, B) Wild type and MOR-KO mice (fed on SED) were killed for intestine sampling. G6Pase activity (**A**) and PEPCK-C protein amount (**B**) determined from a jejunum sample. The data are expressed as the means \pm SEM of 5 mice per group. *, Significantly different from WT mice (p < 0.05).

C) Jejunal G6Pase activity was assayed at the end of the experiment in wild type and MOR-KO mice fed on PED (relating to Figure 6C) and compared to two groups studied in parallel, continuously fed on SED. The data are expressed as the means \pm SEM of 6 mice per group. *, Significantly different from SED fed mice (p < 0.05).

Table S1 (related to Experimental procedures): Antibodies used and conditions of utilization

Antibody	Manufacturer	Use	Dilution
<u>Primary antibodies</u>			
Rabbit anti-mu opioid receptor	Abcam (ab10275)	Immunofluorescence	1:3,000
Mouse anti-PGP9.5	Abcam (ab8189)	Immunofluorescence	1:20
Rabbit anti-c-Fos	Calbiochem (Ab-5)	Immunohistochemistry	1:20,000
<u>Secondary antibodies</u>			
Alexa 488 Donkey anti-Rabbit IgG	Molecular Probes (A21206)	Immunofluorescence	1:2,000
Alexa 555 Goat anti-Mouse IgG	Molecular Probes (A21422)	Immunofluorescence	1:2,000
Biotinylated Donkey anti-Rabbit IgG	Rockland (611-7602)	Immunohistochemistry	1:500

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Binding studies to MORs in N1E115 cells

The N1E115 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 11 g/L sodium pyruvate, supplemented with 15% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/mL) and streptomycin (0.1 mg/mL), in a humidified incubator at 37 °C and 5% CO₂ in air. All culture products were purchased from Life Technologies SAS (Villebon sur Yvette, France). The cells were seeded onto 12-well dishes at a high density (50,000-65,000 cells/cm²) and plated for 48 h.

[Tyrosyl-3-5-³H(N)]-DAMGO (SA: 1.976 TBq/mmol (Perkin Elmer SAS, Courtaboeuf, France) was used to assess peptide binding affinity. After 48-h plating of N1E115 cells, the medium was removed and the cells were incubated for 1 h at 37 °C with 10 nM [³H]-DAMGO, as previously described (Zhang et al., 2006), and increasing concentrations of either unlabeled DAMGO or peptides in DMEM containing 0.5% bovine serum albumin. Binding reactions were stopped on ice by removing the media and quickly washing the cells three times with 0.9% NaCl. The cells were solubilized in 0.5 mL of 0.5M

NaOH and 0.4% sodium deoxycholate, as described previously (Penhoat et al., 1989). The radioactivity of the lysate was quantified in a Packard Liquid Scintillation Analyzer (Perkin Elmer) with 60% efficiency.

Coupling to adenylate cyclase in N1E115 cells

The N1E115 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 11 g/L sodium pyruvate, supplemented with 15% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/mL) and streptomycin (0.1 mg/mL), in a humidified incubator at 37 °C and 5% CO₂ in air. All culture products were purchased from Life Technologies SAS (Villebon sur Yvette, France). The cells were seeded onto 12-well dishes at a high density (50,000-65,000 cells/cm²) and plated for 48 h.

In order to study the coupling of MOR to adenylate cyclase, N1E115 cells were incubated in media containing various metabolites, either alone or in combination at a concentration of 10⁻⁷ M, in the presence of 1 mM 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma-Aldrich) to inhibit the phosphodiesterase activity. After a 20 min-incubation at 37 °C, the cells were recovered, and solubilized (Penhoat et al, 1989), and the intracellular accumulation of cAMP was measured using a radioimmunoassay with ¹²⁵I-labeled cAMP (Beckman Coulter, Roissy, France).

SUPPLEMENTAL REFERENCES

Penhoat, A., Jaillard, C., and Saez, J. M. (1989). Corticotropin positively regulates its own receptors and cAMP response in cultured bovine adrenal cells. *Proc Natl Acad Sci U S A* 86, 4978-4981.

Zhang, J., Frassetto, A., Huang, R. R., Lao, J. Z., Pasternak, A., Wang, S. P., Metzger, J. M., Strack, A. M., Fong, T. M., and Chen, R. Z. (2006). The mu-opioid receptor subtype is required for the anorectic effect of an opioid receptor antagonist. *Eur J Pharmacol* 545, 147-152.

