

Supplementary methods

Intragastric surgical procedures

Under anesthesia (Isoflurane), a catheter was surgically inserted into the stomach. Briefly, a 4mm laparotomy was performed on the left side of the abdomen, and the stomach was gently extracted. One centimeter of a Teflon catheter was inserted into the stomach and secured by surgical glue (Histoacryl; 3M Health Care, St. Paul, MN). The other extremity of the catheter was tunneled under the skin and exteriorized at the back of the neck [1]. After a 1-week recovery period, hypothalamic NO amperometric measurements were performed in mice.

Intracerebroventricular surgical procedures

Under anesthesia (Isoflurane) an indwelling intra cerebroventricular (icv) catheter (Alzet Brain Perfusion Kit 3, 1-3 mm, Charles River, -1mm lateral to the sagittal suture, -0.2mm posterior to the bregma and -1.7mm below the skull surface) was implanted [2].

Acute injections

Apelin. For *in vivo* acute perfusions, 100 μ L of [Pyr]apelin-13 (Bachem, UK), the most active and stable apelin isoform [3, 4], at a concentration of 100nM and 1 μ M, were injected orally or by the intragastric catheter. For *ex vivo* acute perfusions, [Pyr]apelin-13 was injected directly into the survival medium in order to obtain final concentrations from 1pM to 10 μ M.

Apelin receptor (APJ) antagonist. For *in vivo* acute perfusions, 100 μ L of an APJ antagonist, F13A (Polypeptide) [5], at a concentration of 1g/L [6], were administered orally or by the

intra-gastric catheter. F13A was injected alone or with apelin 100nM or 1 μ M. For *ex vivo* acute perfusions, F13A was injected directly in the survival medium in order to obtain final concentration of 1g/L. F13A was injected alone or with apelin 100nM or 1 μ M.

Nitric Oxide Synthase (NOS) inhibitor. For *in vivo* acute perfusions, 2 μ L of NOS inhibitor, L-NG-monomethyl Arginine citrate (L-NMMA, Sigma), at a concentration of 100 μ M were injected directly to the icv catheter completed to 2 μ L of artificial cerebrospinal fluid (aCSF) [2]. The L-NMMA infusion was started 30 min before the apelin oral gavage. For *ex vivo* perfusions, L-NMMA was injected directly in the survival medium in order to obtain final concentration of 100 μ M [2].

Agonist of β -adrenergic receptors. For *in vivo* acute perfusions, 100 μ L of Isoproterenol (Sigma), at a concentration of 0.1 μ M, were administered by the intra-gastric catheter. For *ex vivo* perfusions, Isoproterenol, was injected directly in the survival medium in order to obtain final concentration of 0.1 μ M [7].

Antagonist of nicotinic receptors. For *in vivo* acute perfusions, 100 μ L of Hexamethonium (Sigma), at a concentration of 500 μ M, were administered by the intra-gastric catheter. Hexamethonium was injected alone or with apelin 100nM or 1 μ M. For *ex vivo* perfusions, Hexamethonium, was injected directly in the survival medium in order to obtain final concentration of 500 μ M [8]. Hexamethonium was injected alone or with apelin 100nM.

Capsaicin. Subcutaneous injection at the neck level of Capsaicin (Sigma) was made in two injections: 50mg/kg and 75mg/kg 24h apart under anesthesia, 7 days before the intra-gastric administration of apelin 100nM or control (water). The efficacy of capsaicin pretreatment to ablate sensory nerves was tested just before the sacrifice of the animals, as previously described [9].

Telemetry

Under anesthesia, a needle attached to a catheter was placed in the stomach for intragastric injection (H₂O or drugs including apelin). Two electrodes were sutured 5mm from each other, on the proximal duodenum, 1.5cm caudal to the pylorus. The signal, corresponding to the electrical activity of duodenum, was received by an RMC-1 receiver (DSI) placed under the cage. The signal obtained during 40min of continuous recording was analyzed off-line to obtain the integral of the rectified signal over the 100ms integration interval. The electric signal was allowed to stabilize during 5min. The next 5 minutes were considered as basal signal, and subsequently the mice received H₂O or drugs by intragastric injection of 100μl at a rate of 10μl/min using a syringe pump.

Isotonic contractions

Mice were euthanized in fed conditions. After dissection, duodenum, jejunum and ileum segments were washed in Krebs-Ringer bicarbonate/glucose buffer (pH 7.4) in an atmosphere of 95% O₂ - 5% CO₂. Segments of intestine were then incubated in oxygenated Krebs-Ringer solution for 30 minutes at 37°C and then attached to the isotonic transducer (Lever Transducer, B40 type 373, Hugo Sachs Elektronik) and immersed in Falcon tubes containing 25mL of the same medium maintained at 37°C. The load applied to the lever was 2g (20mN). Isotonic contractions were recorded on BDAS software (Hugo Sachs Elektronik) following the transducer displacement. After attaching of intestine segments, basal contractions were recorded for 10 minutes. Subsequently, 100μL of Krebs-Ringer solution or specific drugs were added in survival medium and contractions were recorded for 10 minutes. Amplitudes were recorded for 10 minutes at 10-second intervals and their average

was compared to the average basal contractions. Contractions amplitudes are presented as percentage relative to the basal response whilst contraction frequencies are presented as number of contraction per minute [7]. For the one-week chronic apelin gavage protocol, basal contractions were recorded for 5 minutes and are presented as average basal contractions.

Immunohistochemistry

Immediately after dissection, duodenal tissues were fixed in 4% formalin solution (Sigma) for 24h and maintained at 4°C in 70% ethanol until paraffin embedding. Ten-micrometer-thin sections were incubated with goat anti-nNOS (1/200, ab1376, Abcam), goat anti-ChAT (1/100, AB144P, Millipore) and rabbit anti-APJ (1/100, ab140508, Abcam) primary antibodies for 12 hours at 4°C. After washing with PBS 1X, sections were incubated with Cyanine 3-labeled anti-Goat (1/50, 395570, Interchim) and Dylight 488-labeled anti-rabbit (1/100, FES062, Interchim) secondary antibodies for 2 hours at 4°C. Sections were washed with PBS 1X and then incubated with DAPI (D8417, Sigma) (1/1000 in PBS 1X) for 1h at 4°C. After a final washing step, sections were mounted with Mounting media (Sigma). Samples were imaged with a Zeiss LSM 710 confocal microscope (Jena, Germany) using the 63X oil objective (NA 1,4).

Transcytosis

Mice were anesthetized with isoflurane. A small longitudinal incision of the abdomen was made and a loop of the duodenum was exposed. The duodenum was clamped below the

pyloric sphincter and another clamp was installed downstream to the first one in order to delineate a chamber of 2cm long. H₂O or Apelin-TAMRA (1μM; Apelin 13 conjugated to the fluorescent dye TAMRA (5-Carboxytetramethylrhodamine) on its N-terminal part was from Proteogenix (Oberhausbergen, France) was then injected inside the duodenal chamber. Apelin-TAMRA is a functional apelin molecule, which is able to bind to the apelin receptor APJ. Five minutes after the injection, duodenal tissues were collected. Duodenal tissues were included in formalin solution (SIGMA) for 24h, then in 20% sucrose solution for 48h and frozen at -80°C in Tissue-Tek (Polyfreeze, Sigma). Ten-micrometer-thin cryostat sections were made. Sections were washed with PBS 1X and then incubated with DAPI (D8417, Sigma) (1/1000 in PBS 1X) for 1h at 4°C. After a final washing step, sections were mounted with Mounting media (Sigma).

Cell culture and fluorescence microscopy

HEK-293T cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin (Gibco, Saint Aubin, France) in a 5%CO₂ humidified incubator at 37°C. For transient transfection, 3x10⁶ cells were seeded in a 100mm dish. 12 hours later, cells were transfected with 1μg of pEYFP-hAPJ using Calcium Phosphate protocol as previously described [10]. The day after, the cells were detached and seeded on fibronectin coated glass slides in 12-well dishes (2x10⁵ cells/well). 48 hours post transfection, cells were stimulated or not with apelin-TAMRA (100nM) for 1 hour at 37°C and then fixed for 15 min. in 4% paraformaldehyde in PBS. Slides were mounted in fluorescent mounting medium

(Dako, Carpinteria, CA, USA) and images were taken using a Zeiss LSM-780 confocal microscope using a 63X objective. Apelin-TAMRA is a functional apelin molecule which is able to bind and activate its receptor APJ (supplementary figure S1).

Acetylcholine release

Mice were euthanized in fed conditions. After dissection, duodenum fragments were washed in Krebs-Ringer bicarbonate/glucose buffer (pH 7.4) in an atmosphere of 95% O₂ - 5% CO₂ and then immersed in Eppendorf tubes containing 400µL of the same medium. After 30 minutes, 300µL of medium were collected and immediately frozen for basal release of acetylcholine quantification. Then 300µL of Krebs-Ringer or drugs were added in Eppendorf tubes containing duodenum fragments. After 30 minutes, 300µL of medium were collected and immediately frozen for acetylcholine release quantification. Acetylcholine release was measured with commercially available ELISA kit (Abcam).

Oral glucose tolerance test (OGTT)

Six-hour-fasted mice were orally loaded with glucose (3g/kg of body weight). Blood was collected from the tail vein at -30, 0, 15, 30, 60, 90, and 120min and glycemia measured with a gluco-meter (Accu-Chek Active, Roche) [1].

Insulin assay

Serum insulin was measured using an ultra-sensitive mouse insulin ELISA (Merckodia).

Apelin assay

For *in vivo* experiments, six-hour-fasted mice were orally loaded with glucose (3g/kg of body weight) alone or with drugs. Mice were euthanized 5 minutes after this oral gavage and duodenum, jejunum and ileum were harvested and immediately frozen for subsequent apelin quantification studies. For *ex vivo* experiments, two-hour fasted mice were euthanized. The duodenum was then harvested, washed, everted, and filled with in a Krebs-Ringer solution without glucose. Everted duodenal sacs were incubated in Krebs-Ringer with 10g/L of glucose alone or with drugs, for 2 minutes at 37°C. Tissues were then collected and immediately frozen for subsequent apelin quantification studies. Apelin was measured with a commercially available ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA).

Glucose and apelin absorption

Two-hour fasted mice were euthanized. The duodenum was then harvested, washed, everted, and filled with in a Krebs-Ringer solution without glucose. Everted duodenal sacs were incubated in Krebs-Ringer with 10g/L of glucose alone or with drugs, for 2 minutes at 37°C. The media of each sac and was then collected and immediately frozen for subsequent apelin and glucose quantification studies. For portal vein experiments, mice were euthanized 10 minutes after an oral gavage of H₂O or drugs. Blood was harvested from the portal vein. Serum was then collected and immediately frozen for subsequent apelin quantification studies. Apelin was measured with a commercially available ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA). Glucose was measured using glucose RTU™ kit (bioMérieux).

Glucose utilization

Two-hours-fasted mice were orally loaded with 50 μ L of [3-³H]glucose (Perkin Elmer) and 100 μ L of H₂O or drugs. Thirty minutes after the oral load, mice were euthanized and blood, liver, muscle and adipose tissue were collected, precisely weighed and immediately frozen. Tissues were dissociated in NaOH 1M (50, 200 and 200 μ L for muscle, liver and adipose tissue respectively) during 1 hour at 55°C. Samples were centrifuged at 7000g during 5 minutes. Fifty μ L were taken from supernatant and added to 3mL of Emulsifier-Safe™ (Perkin-Elmer). The radioactivity was counted as dpm. Data are presented according to following formula $\text{Log}_{10}(\text{dpm/g tissue})$.

Metabolic parameters

Insulin resistance index was determined by multiplying the area under the curve (-30 min and 15 min) of both blood glucose and plasma insulin obtained after an OGTT as previously described [11]. Homeostasis model assessment insulin resistance (HOMA-IR) was determined by using the following adjusted formula: $\text{HOMA-IR} = \text{fasting Glucose (mmol/L)} \times \text{fasting Insulin (mU/L)} / 22.5$.

qPCR

Total RNAs were extracted from muscle using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA (500 ng) was reverse transcribed for 60 min at 37°C using Superscript II reverse transcriptase (Invitrogen, St Aubin, France) in the presence of random hexamers. Real-time PCR was performed on 12.5 ng cDNA and 100–900 nmol/l specific oligonucleotides

primers in a final volume of 20 µl using the Mesa blue qPCR Master Mix for Sybr (Eurogentec, Angers, France). Fluorescence was monitored and analysed in a StepOnePlus Real-Time PCR system instrument (Applied Biosystems, St Aubin, France). Beta2microglobulin RNA was used to normalize gene expression according to the following formula $2^{-(Ct_{\text{Beta2microglobulin}} - Ct_{\text{gene}})}$ where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold. Sequences of the oligonucleotides primers are : Apelin sense 5'- GTTTGTGGAGTGCCACTG -3' and antisense 5'- CGAAGTTCTGGGCTTCAC -3' ; APJ sense 5'- GCTGTGCCTGTCATGGTGTT -3' and antisense 5'- CACTGGATCTTGGTGCCATTT -3' ; GLUT-4 sense 5'- CCGGATTCCATCCCACAAG -3' and antisense 5'- CATGCCACCCACAGAGAAGA -3' ; Beta2microglobulin sense 5'- CACTGACCGGCCTGTATGC -3' and antisense 5'- GGGTGGCGTGAGTATACTTGAATT -3'.

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