Supplemental Methods

Cell culture

Primary cultures of tanycytes of the median eminence. Tanycytes were isolated from the median eminence of the hypothalamus of 10-d-old rats and cultured as described previously (1, 2). Briefly, after decapitation and removal of the brain, median eminences were dissected and crushed on 80µM nylon mesh (Sefar America Inc., Kansas City, MO). Dissociated cells were cultured in DMEM/F12 (Invitrogen, Cergy Pontoise, France) supplemented with 10% (v/v) donor calf serum (Invitrogen) under humid atmosphere of 5 % CO_2-95 % air at 37 °C. Culture medium was changed after 3-4 days of culture and subsequently every 2 days. Upon reaching confluence, the tanycytes were isolated from contaminating cells by overnight shaking at 250 rpm at 37 °C and either replated in 10 cm dishes for Western Blot experiments or seeded in culture plates on poly-L-lysine-coated glass coverslips for studying actin cytoskeleton remodeling and coculture experiments. Two days before treatment, the medium was replaced by a tanycyte/endothelial defined medium (TEDM) consisting of DMEM/F12 (devoid of phenol red; Invitrogen) supplemented with insulin (5µg/ml) (Sigma, Saint Quentin Fallavier, France) and putrescin (100µM) (Sigma).

Purification of endothelial cells of the median eminence by sequential immunopanning. Vascular endothelial cells of the median eminence (ECME) were isolated from 10-d-old rats using a procedure previously described (1). Briefly, after papain (Worthington/Cooper, Lakewood, NJ) dissociation of median eminence explants in MEM/Hepes (Invitrogen) containing L-cysteine (0,4mg/ml) (Sigma) and DNase (125units/ml) (Sigma), tissues were triturated in a solution containing ovomucoid trypsin inhibitor solution (2mg/ml) (Boehringer-Mannheim, Mannheim, Germany), DNase (125 units/ml) and BSA (1mg/ml) (Sigma) to get a suspension of single cells. The suspension was filtered through a 20µm nylon mesh. After centrifugation at 550g, single cells were successively panned on a first Petri dish coated with an anti-CD90 mouse monoclonal antibody (MRC-OX7, Serotec, Oxford, UK), on a second

Petri dish coated with RAN-2 ascites (LGC Promochem, Molsheim, France); and the remaining cells were incubated on a third Petri dish coated with *Bandeiraea simplicifolia* Lectin II (BSL II) (Vector Laboratory, Burlingame, CA). The purified endothelial cells were cultured in DMEM supplemented with 10% of fetal bovine serum, 1 % L-glutamine and 1 % penicillin/streptomycin until they reached confluence. Then they were recovered by trypsin digestion from the last panning dish and plated in 10 cm dishes for Western Blot experiments or seeded in 12 well-plates for coculture experiments. Cells were cultured in TEDM 2 days before being used for experiments.

Coculture of tanycytes with endothelial cells of the median eminence.

Tanycyte cultures seeded onto 18mm diameter coverslips coated with poly-L-lysine (Sigma) were grown until they reached 90% confluence. After 2 days of culture in TDM in the presence or absence of physiological levels of 17β-estradiol (5 nM) (3, 4), the coverslips were placed above a confluent monolayer of vascular endothelial cells that had or had not been exposed to estradiol for 48h, respectively, with the tanycyte cell layer facing towards the endothelial cells and separated from them by glass chips. Cells were cultured for 30 min in fresh TDM. The coverslips were then fixed in 4% paraformaldehyde for the visualization of the actin cytoskeleton of the tanycytes. In some cocultures, endothelial NO release was inhibited or further stimulated by preincubating the endothelial cells with 1mM N_{ω}-Nitro-L-arginine methyl ester (L-NAME; Sigma), a nitric oxide synthase (NOS) inhibitor, or 500 µM L-arginine (Sigma), the precursor of NO, respectively, for 30 min.

Cell treatment

To determine the effect of estradiol on NO-mediated actin cytoskeleton remodeling in tanycytes, estradiol-pretreated (5 nM, 48h) tanycyte cultures were exposed to 10, 100 or 1000nM concentrations of sodium nitroprusside (SNP; Sigma), a NO donor.

To test whether the highest concentration of SNP (1000nM) is toxic to cultured tanycytes, cells were treated for 30 min and then rinsed and cultured in fresh TDM medium

for an additional 24 h. Cells were then fixed and submitted to an *in situ* cell death detection kit using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) according to manufacturer's instructions (Roche, Manheim, Germany). Positive controls for TUNEL staining consisted of fixed-cell cultures treated with 10 µg/ml DNase I for 10 min at room temperature to induce DNA strand breaks. Fluorescein-dUTP labeling of DNA strand breaks was visualized with a Leica TCS SP confocal system, with a 25 X NA 0.75 PL FLUOTAR objective, using the 488 nm line of an argon gas laser. The intensity of the laser beam and the sensitivity of the photoreceptor were kept constant to allow comparisons between treatments. At least five optical fields per culture were examined.

To determine whether the application of cyclooxygenase (COX) or soluble guanylate cyclase (sGC) activity products mimic the effects of estradiol on NO-mediated actin cytoskeleton remodeling in tanycytes, cultured tanycytes were treated with prostaglandin E2 (PGE_2, 2.8nM, 280nM or 1 µM, Calbiochem, Meudon, France) and/or 8-Bromo-cyclic GMP (Br-cGMP, 2.8nM or 280nM, Calbiochem), a cell permeable cGMP analog, respectively, for 30 min.

Fluorescent staining

After treatment, cells were fixed with paraformaldehyde (4%) in PBS and rinsed twice. Cells were permeabilized with 0.1% triton X-100 for 5 min and then stained with Alexa 588® -X phalloidin (Molecular Probes, Eugene, OR) diluted 1:40 in PBS, for 45 min at room temperature. Phalloidin is a toxin that specifically recognizes filamentous actin. Culture slides were rinsed in PBS and cell nuclei were stained with Hoechst (Molecular Probe, Eugene, OR).

Determination of actin cytoskeleton remodeling

For morphological evaluation of the reorganization of the actin cytoskeleton in isolated tanycytes submitted to different treatments, cells were classified into 3 different phenotypic classes, defined as follows. Class 1: cells with "cortical actin", i.e. actin present around the

edges of the cell, and that can be considered submembranous actin; class 2: cells bearing heavy stress fibers, parallel actin fibers present throughout the cytoplasm, but no cortical actin; class 3: cells bearing retracted cell processes. Alexa 588® -X phalloidin-stained cultures were imaged using a fluorescent system (DMRB microscope, DC300FX camera, FW4000 software; Leica). Twelve fields per coverslip, chosen at random, were acquired at 400 X magnification. For each image, the total number of cells present in each field was counted. Then, cells corresponding to each class were counted sequentially. With this method, each cell was counted only once and thus reported in only one group. The results obtained from sequential analysis of the same field were added, and the percentage of cells belonging to each class calculated. At least 4 coverslips were analyzed per experimental condition and the results averaged. Each experiment was repeated at least twice using independent cultures. To avoid bias on the part of the observer, the quantification was repeated by an independent investigator blind to the experimental conditions.

Immunoprecipitation

Infected and uninfected endothelial cells of the median eminence in 12-well plates were rinsed with ice-cold PBS and snap frozen on dry ice. Two hundred microliters of fresh lysis buffer (25 mM Tris pH 7.4, 50 mM β-glycerophosphate, 1 % triton X-100, 1.5 mM EGTA, 0.5 mM EDTA, 1mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 µg/ml leupeptin and pepstatin A, 10 µg/ml aprotinin, 100 µg/ml PMSF) were used to extract proteins in each well. Three wells were pooled and the cell lysates were cleared by centrifugation at 12,000 X g for 15 min. Two micrograms of the rabbit polyclonal IgG raised against the C-terminus of eNOS (sc-654, Santa Cruz, CA), recognizing the native protein but not the truncated form, in a total volume of 750 µl of lysis buffer were incubated with gentle rocking overnight at 4°C. Thereafter, 60 µl of protein A-Sepharose beads (Sigma) in lysis buffer (1:1 blend) was added to each sample and incubated for 6 additional hours with gentle rocking at 4°C. The Sepharose beads were collected by centrifugation, washed twice with ice-cold lysis buffer

and boiled for 5 min in 50 µl of 2X sample buffer (187 mM Tris-Base, 9 % SDS, 15 % glycerol, 15 % β-mercaptoethanol and bromophenol blue 86 µM, pH 6.8). Samples were stored at -80 °C until use.

Western Blotting

For straight Western blotting, estradiol-treated or -untreated cells in 10cm dishes or cells subjected to viral infection in 12-well plates were rinsed with ice-cold PBS and snap frozen on dry ice. Cells were then quickly thawed and proteins were immediately extracted with fresh lysis buffer. The protein content of cell extract was determined using Bradford method (BioRad, Hercules, CA). Extracted and/or immunoprecipitated protein samples stored at -80°C in sample buffer were boiled for 5min after thawing and size-fractionated in 4-20% polyacrylamid-SDS precast gels (Invitrogen, Carslab, CA). After electrophoresis the proteins were transferred onto polyvinylidene difluoride membranes (PVDF; Invitrogen) for 3h on ice at room temperature. The membranes were blocked in 5% non fat milk in Tris buffer saline for 1h at room temperature and subjected immunoblotting using the rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues around Ser600 of eNOS (1:1000; #9572, Cell Signaling Technology) and thus recognizing both native and truncated forms of eNOS, soluble GC polyclonal rabbit antibody (1:1000; #371712; Calbiochem), COX I and COX II polyclonal goat antibodies (1:500; sc-1752 and sc-1745, respectively, Santa Cruz), ERα polyclonal rabbit antibodies (1:500; #06-935, Upstate, Waltham, MA) and ERβ polyclonal goat antibodies (1:100; sc-6822; Santa Cruz), overnight at 4°C. To develop the immunoreaction, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma) for 1h at room temperature, and developed using enhanced chemoluminescence (NEN, Boston, MA).

Assessment of ultrastructural changes induced by PGE_2 treatment in median eminence tissue

To determine whether PGE_2 promotes morphological changes in the external zone of the median eminence, *ex vivo* experiments were performed using a protocol described previously (1). Female rats weighting 230-250 g with regular estrous cycles were killed by decapitation on the day of diestrus 2, and the hypothalamus containing the median eminence was microdissected. Hypothalamic tissue was preincubated for 30 min at 37°C in 2 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 4.5 mg/ml D-dextrose, and 5 μM tetrodotoxin to inhibit neurotransmitter release through the activation of voltage-gated sodium channels, in an air atmosphere containing 5 % CO_2. After this preincubation period, the tissue was placed in fresh medium containing 1 μM PGE_2 (treated group; n = 4), or not (control group; n = 4), for an additional 30 min incubation period. Explants were subsequently processed for electron microscopy as described previously (5, 6). Briefly, the tissue was fixed by immersion in a mixture of 2% paraformaldehyde, 0.2% picric acid and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. The tissue was postfixed for 1 h at room temperature with 1% OsO_4 in phosphate buffer. After dehydration, pieces of tissue were embedded in Araldite. Semithin sections (1-2 μm thick) were used to progressively approach and identify the portion of the median eminence where the ultrastructural studies were to be carried out, i.e. the area where the pituitary stalk becomes distinct from the base of the hypothalamus but still remains attached to it by the hypophyseal portal vasculature system (5, 6). This area, which does not extend more than 20 μm, contains high numbers of GnRH fibers. To detect GnRH immunoreactivity, ultrathin sections (80-90 nm thick) collected on 100 mesh grids coated with parlodion 0.8%-isoamyl acetate (EMS, Fort Washington, PA) were treated using an immunogold procedure described previously (5, 6). The sections were also counterstained with uranyl acetate and lead citrate before observation. Immunostained ultrathin sections were examined with a Zeiss transmission electron microscope 902 (Leo, Rueil-Malmaison, France) and images were acquired using a Gatan Orius SC1000 CCD camera (Gatan France, Grandchamp, France).

Analysis was performed on 10-15 ultrathin sections per animal, with a space of 25 sections between them, to avoid taking the same GnRH nerve terminal into consideration

twice (the diameter of a GnRH nerve terminal rarely exceeds 2 µm). All immunolabeled terminals confined to a distance of 5 µm or less from the basal lamina were imaged and the distance from the nerve terminal and the pericapillary space recorded.

Intracerebral infusion of an inhibitor of COX activity

To determine the importance of functional COX activity within the median eminence for the central control of the reproductive cycle, *in vivo* experiments were performed. Indomethacin, an inhibitor of COX activity, was chronically infused into the median eminence of the brain (Bregma -3.6 mm, 9.5 mm deep from the surface of the skull) (7) through a stereotaxically implanted infusion cannula (Plastic One, Roanoke, VA) connected to a subcutaneously implanted Alzet mini-osmotic pump (Alzet Corporation, Palo Alto, CA). The pumps (model 1007D) have a flow rate of 0.5 µl/h and a capacity of 100 µl, resulting in a delivery period of 7 days. Each pump was loaded with 1% ethanol in sterile 0.9% NaCl, with or without 560 µM Indomethacin, connected to the infusion device and primed overnight in 0.9% NaCl at 37°C. Each assembly was implanted into a 230-250 g rat with a regular estrous cycle. The estrous cycle was monitored before and after surgery by daily inspection of vaginal cytology: diestrus 1 and 2 were defined by the presence of a predominance of leukocytes in the vaginal smears, the day of proestrus was characterized by a predominance of round nucleated epithelial cells in vaginal smears and the day of estrus was distinctively characterized by large numbers of cornified squamous epithelial cells, which occur in clusters. Subsequent to the infusion experiment, animals were killed to confirm the implantation site of the cannula and to assess the level of exhaustion of the infusion solution.

Supplemental References

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