Supporting Information

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SI Materials and Methods

Reagents. The anti-Oxtr antibody has been described previously (1). All other chemicals, including tissue culture reagents, were purchased from Sigma Chemical Co. unless otherwise indicated.

Calvarial Cell Culture. Osteoblast-enriched cultures were performed using calvaria from newborn mice, which underwent sequential collagenase digestion. Cells were grown in α-modified MEM (αMEM) supplemented with 10% (vol/vol) FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Osteoblast-enriched calvarial cells were seeded at the density of 20,000 cells/cm^2 for each experiment.

Nuclear Isolation. Intact nuclei were isolated as described by Adebanjo et al. (2). Cells were homogenized in cold Tris·HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.25 mM sucrose (TKM/sucrose) solution. The homogenate was then centrifuged (700 × *g* for 10 min), and the pellet was homogenized in PBS two times (five strokes each time) and centrifuged at 700 × *g* after each homogenization step (all at 4 °C). The resulting nuclear preparations were resuspended in a medium containing 0.25 M sucrose, 10 mM $MgCl_2$, 1 mM DTT, and 50 mM Tris·HCl (pH 7.4). Sodium citrate (1% wt/vol) was added to this nuclear suspension, which was incubated for 30 min on ice while stirring gently followed by centrifugation for 15 min at 500 × *g*. The supernatant consisted of the outer nuclear membrane, and the pellet contained nucleoplasts (nuclei without outer membranes) (2). Nucleoplasts were lysed for protein analyses or seeded on glass coverslips, fixed, and stained for microscopic observation.

Immunofluorescence Microscopy. Cells were counted and seeded on glass coverslips, grown in αMEM [10% (vol/vol) FBS], and fixed in 4% (vol/vol) paraformaldehyde/PBS. The fixed cells or nuclei were washed with PBS and blocked in 1% BSA and 5% (vol/vol) normal goat serum in PBS for 20 min. Samples were incubated with antibodies to Oxtr (gift from Fabio Malavasi, University of Turin, Turin, Italy), Arrb1/2 (Santa Cruz Biotechnology), Tnpo1, Kpnb1 (Abnova), and Rab5 (Cell Signaling). After washing, bound antibodies were detected using 2 μg/mL fluorescent-labeled goat anti-mouse or anti-rabbit secondary antibody (Alexa-488 and Alexa-568, respectively; Invitrogen). Nuclei were counterstained with Quinolinium 4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-diiodide (TO-PRO). Cells were then visualized and photographed using a multispectrum confocal microscope (Leica TCS SP5).

Immunogold Staining. Cultures were fixed in 0.1 M phosphate-buffered 0.5% glutaraldehyde for 30 min. After rinsing with 10 mM ammonium chloride in 0.1 M PBS for 45 min, the cells were dehydrated in ascending ethanol [up to 70% (vol/vol)] in acrylic resin LR-Gold (London Resin) with 0.8% SPI-ChemT Benzil (London Resin). The resin was hardened at −25 °C under a 500-W halogen lamp; 60-nm thin sections, previously identified on toluidine blue-stained semithin sections, were cut with an LKB V ultratome and collected on formvar-coated nickel grids. The grids were incubated for 10 min at room temperature with Tris-buffered saline (TBS) and then treated with 1% BSA–TBS (pH 7.4) for 10 min at room temperature (to block nonspecific reactions). Sections were incubated at room temperature overnight with primary mouse anti-Oxtr (1:30), washed with TBS, and incubated for 1 h at room temperature with secondary antibodies (goat anti-mouse IgG coupled with 30- or 20-nm gold particles). After washing with TBS, grids were stained with 1% uranyl acetate followed by 1% lead citrate and examined with a Zeiss EM 109 electron microscope. Digital images were obtained with a cooled camera (Gatan CMS; Gatan GMbH). Preimmune mouse serum (Dako) replacing the primary antibodies served as a negative control.

Immunoblotting. Cytosolic and nuclear fractions were separated (2). Thereafter, nuclear protein was prepared per the manufacturer's instructions (78833; Thermo Scientific). Total protein was measured using the Bio-Rad Protein Assay Kit, and both fractions were separated by SDS/PAGE before transfer onto nitrocellulose membranes (Invitrogen). After immunoblotting with the appropriate antibodies, immune complexes were incubated with IRDye-labeled secondary antibodies (680/800 CW) and visualized using the Odyssey infrared imaging system (LI-COR).

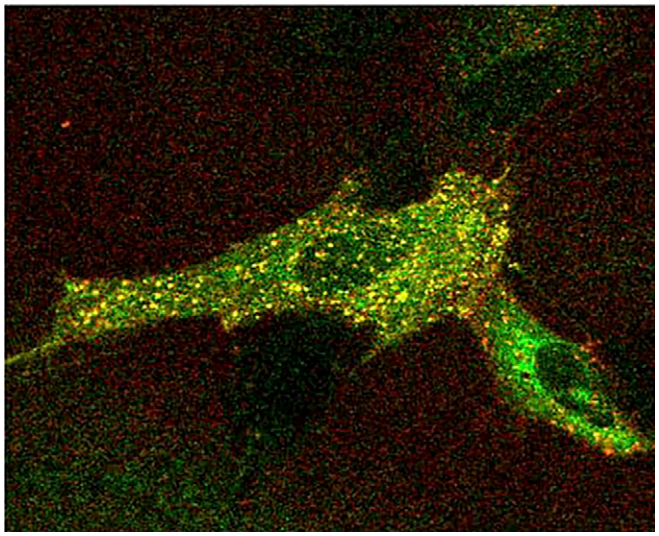
MALDI-TOF MS. Nuclear proteins were immunoprecipitated with anti-Oxtr antibody and separated by SDS/PAGE. The band corresponding to Oxtr was cut, and tryptic digestion was performed using RapiGest. The solution obtained from tryptic digestion was spotted on the target plate. After drying, the spot was analyzed using a Micromass MALDI-TOF Mass Spectrometer (Waters MS Technologies). Positive ion spectra were acquired in reflectron mode using the following voltages: pulse, 2,610 V; source, 15,000 V; reflectron, 2,000 V; microchannel plate, 1,900 V. The laser firing rate was 5 Hz, and 80 laser shots were used. The resulting spectra were averaged, background-subtracted, and smoothed by a Savitzky–Golay algorithm. For protein identification, the raw files derived in gel tryptic digestion were searched against the nonredundant database Swiss-Prot using Aldente database search engines and ExPASy FindPept. The experimentally measured peptide masses are compared with the theoretical peptides calculated from a specified Swiss-Prot/TrEMBL entry; this comparison means that only one entry at a time can be considered. In our case, *Oxtr* was selected as the specific entry.

siRNA Knockdown. Primary mouse calvaria osteoblasts were plated in 35-mm dishes in αMEM [10% (vol/vol) FBS] and transiently transfected with *Arrb1*, *Arrb2*, or *Tnpo1* siRNA (50 nM) using RNAi Max Lipofectamine (Life Technologies). Significant knockdown (70–80%) of proteins was obtained 48–60 h posttransfection, at which time cells were stimulated with Oxt and lysed for RNA and protein analysis.

Mutagenesis. Oxtr contains three Ser clusters in the C-terminal tail that are targets for phosphorylation by GPCR kinases and Arrb interactions. It has been shown that mutations in two of three Ser clusters destroy interaction with Arrb (3). A mouse Oxtr mutant construct was generated that had two clusters of Ser mutated to Ala residues (Oxtr^{mut}; PrimmBiotech). The *pcDNA3.1–Oxtr^{wt}* generated by the same company served as the template vector. The resultant plasmid contained S365A, S366A, S367A, S376A, S377A, and S378A point mutations and was named *pcDNA3.1–Oxtr^{mut}*. Positive *Oxtr^{mut}* clones were selected with ampicillin and further purified by Maxi-Prep (Qiagen). Oxtr$^{-/-}$ mouse osteoblasts were stably transfected with *pcDNA3.1–Oxtr^{mut}* or *pcDNA3.1–Oxtr^{wt}* using Lipofectamine 2000 (Life Technologies) and selected with geneticin. Geneticin-resistant cells were amplified and used for immunofluorescence, immunoblotting, and quantitative PCR.

Quantitative RT-PCR. Total RNA was isolated (RNeasy; Qiagen) and reverse-transcribed (2 μg; Superscript First-Strand Synthesis Kit; Life Technologies) per the manufacturer's instructions. The resulting cDNA (20 ng) was subjected to quantitative PCR using SYBR Green in a CFX96 Real-Time Thermal Cycler (Bio-Rad). The mean cycle threshold value from triplicate samples was used to calculate gene expression after normalizing to GAPDH levels.

1. Tamma R, et al. (2009) Oxytocin is an anabolic bone hormone. *Proc Natl Acad Sci USA* 106(17):7149–7154.
2. Adebanjo OA, et al. (1999) A new function for CD38/ADP-ribosyl cyclase in nuclear Ca2+ homeostasis. *Nat Cell Biol* 1(7):409–414.
3. Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG (2001) Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis*. *J Biol Chem* 276(22):19452–19460.



Movie S1. Agonist binding triggers Oxtr intracellular trafficking. Shown is a time-lapse video of primary *Oxtr*$^{-/-}$ osteoblast transfected with *Oxtr–EGFP* (green), which appeared at the cell surface and in the cytosol. When the Oxtr agonist dLVT–Alexa-546 (red) was added, diffuse red fluorescence was detectable in the medium. On agonist binding to Oxtr on the membrane surface, yellow vesicles were visible in the cells within 2 min, indicating the internalization of dLVT–Alexa-546–bound Oxtr–EGFP. At 10 min, all of *Oxtr–EGFP* was sequestered into cytoplasmic vesicles.

Movie S1