

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Teles MG, Bianco SDC, Brito VN, et al. A *GPR54*-activating mutation in a patient with central precocious puberty. *N Engl J Med* 2008;358:709-15.

Supplementary Methods

Hormone Assays

Serum LH, FSH, and estradiol levels were measured by immunofluorometric assays (Delfia, Wallac, Inc., Turku, Finland). The coefficient of variation was $\leq 5\%$ for all assays. The lower limits of detection were 0.6 IU/L for LH, 1.0 IU/L for FSH, and 13 pg/mL (47 pmol/L) for estradiol¹¹. Serum LH and FSH were measured at -15, 0, 15, 30, 45 and 60 min after intravenous administration of 100 μ g GnRH and two hours after the first subcutaneous dose of 3.75 mg depot leuprolide acetate. The results were compared with normal values established in our population and with those previously reported^{11, 13}.

Inositol Phosphate (IP) Assays

Twenty-four hours prior to the IP assay, media on transfected COS-7 cells were replaced with inositol-free DMEM for 2 hours at 37°C, after which 2 μ Ci/ml – myo-[2-³H]-inositol (Perkin Elmer, Waltham, MA) was added, followed by the addition of 10 mM LiCl 15 min later. After overnight incubation, IP production was stimulated by the addition of 10^{-11} to 10^{-7} M kisspeptin-10 for the indicated times. Cells were extracted with formic acid, lysates neutralized, centrifuged and protein content determined. Supernatants were loaded onto previously equilibrated AG-X8 resin anion exchange columns (Fisher Scientific, Pittsburgh, PA). The columns were washed and total IP was eluted. Radioactivity was quantified by liquid scintillation and corrected for protein content. All assay points were performed in duplicate or triplicate, and each experiment was repeated at least three times.

Measurement of ERK Phosphorylation

Twenty-four hours after transfection, COS-7 cells were stimulated with 10^{-9} M kisspeptin-10 for the indicated times, after which cells were lysed in RIPA buffer in the presence of 0.1 mg/ml PMSF and 1 mM sodium orthovanadate. Cell lysates were sonicated for 20 s on ice and centrifuged for 10 min at 12,000 g at 4°C. Ten μ g of supernatant protein from each sample was separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated overnight with a mouse anti-pERK IgG (1:5,000; SC-7383, Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with goat anti-mouse IgG-HRP (1:5,000; SC-2060, Santa Cruz Biotechnology). Immunoreactive bands were detected using luminol chemiluminescence reagent (Perkin Elmer) and pERK bands were normalized to total ERK, determined in the same membranes.

Receptor Binding Assays

Forty-eight h after transfection, COS-7 cells were incubated with 100,000 cpm of 125 I-kisspeptin-10 (Perkin Elmer) in the presence of 10^{-10} to 10^{-5} M unlabeled kisspeptin-10 in DMEM containing 10mM HEPES and 1% BSA for 20 min at room temperature. Cells were rinsed 6 times with PBS containing 0.5% BSA and then lysed with 0.2 M NaOH, 0.5% BSA. Cell lysates were collected and radioactivity was measured in a gamma counter. The dissociation constant (Kd) and maximal binding capacity (MBC) were calculated based on nonlinear regression of homologous competition binding analysis using Prism 3.0 (GraphPad Software, San Diego, CA).

Receptor Binding Time-Course Assays

Forty-eight hours after transfection, COS-7 cells were incubated with 100,000 cpm of ^{125}I -kisspeptin-10 (Perkin Elmer) in DMEM containing 10 mM HEPES and 1% BSA. After 4 h on ice, the cells were transferred to 37°C for 0-120 min. Non-specific binding assays for each time-point were run in parallel, in the presence of 10^{-5} M unlabeled kisspeptin-10. Cells were rinsed 3 times with PBS containing 0.5% BSA and then acid-washed for 3 min on ice with PBS + 0.5% BSA + 50 mM acetic acid. The membrane-bound ^{125}I -kisspeptin acidic fraction was then collected and counted in a gamma counter. Specific counts were calculated and plotted using Prism 3.0 (GraphPad Software, San Diego, CA).

Statistical Analysis

The *in vitro* assays were analyzed by two-way ANOVA followed by the Bonferroni post-test for comparison analysis. EC_{50} and V_{max} values were calculated for the dose-response curves using GraphPad Prism (GraphPad Software, San Diego, CA) for non-linear regression sigmoid curves. Results were considered significant when $p < 0.05$.

Supplementary Results

Receptor Binding Characteristics

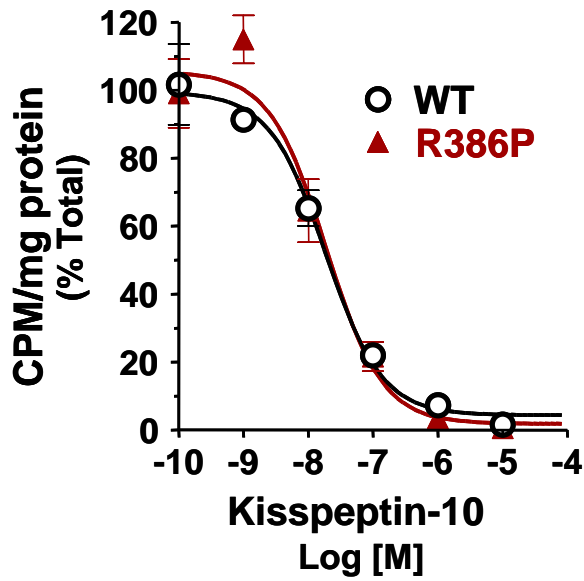
To measure kisspeptin binding by displacement analysis, COS-7 cells transfected with either WT or R386P GPR54 were incubated with ^{125}I -kisspeptin-10 in the presence of 10^{-10} to 10^{-5} M unlabeled kisspeptin-10, and after nonlinear regression of homologous competition binding analysis, the dissociation constant

(K_d) and maximal binding capacity (MBC) were calculated (**Fig. S1**). The displacement curve for R386P was virtually identical to that for WT GPR54 with no significant differences in MBC or K_d , indicating that the R386P substitution does not affect receptor expression levels or affinity for kisspeptin.

Receptor Binding Time-Course Analysis

After allowing equilibration on ice of ^{125}I -kisspeptin-10 binding to COS-7 cells transfected with either WT or R386P GPR54, cells were transferred to 37°C for increasing times to determine the kinetics of cell surface binding. In a representative experiment (**Fig. S2A**), the amount of membrane-bound ^{125}I -kisspeptin at baseline was not significantly different for R386P GPR54 compared to WT receptor, indicating similar levels of cell surface expression. However, following incubation at 37°C, levels of membrane binding were higher in cells transfected with the R386P GPR54 mutant at all time-points tested. These results were consistent and reproducible, as demonstrated in **Fig S2B**. The ratio of R386P:WT ^{125}I -kisspeptin cell surface membrane binding, as determined by combining data from 5 independent experiments, increases over time, reaching 2.5 by 120 min of incubation at 37°C. These results suggest that the R386P mutant receptor remains capable of binding ligand on the cell surface plasma membrane for longer periods of time, extending the window of stimulation in response to kisspeptin exposure.

Figure S1. Displacement binding assay. COS-7 cells were transiently transfected with WT or R386P GPR54 and incubated for 20 min at room temperature with ^{125}I -kisspeptin in the presence of 10^{-10} to 10^{-5}M unlabeled kisspeptin. Each point is the mean \pm SE of 3 replicates.



	K_d (nM)	Maximal Binding (nmol/mg)
WT	4.4	20
R386P	7.0	20

Figure S2. Receptor binding time-course. Time-course of ^{125}I -kisspeptin binding to cell surface plasma membranes of COS-7 cells transfected with WT or R386P GPR54. Non-specific binding for each time-point was measured by incubation with 10^{-5} M unlabeled kisspeptin-10 and subtracted from the total counts at the corresponding time-point to determine specific counts. Specific counts were corrected for protein content. (A) Representative time-course assay showing membrane-bound ^{125}I -kisspeptin-10 in COS-7 cells transfected with WT or R386P GPR54; (B) R386P:WT ratio of membrane-bound ^{125}I -kisspeptin-10. Each bar represents the mean \pm SEM of 5 independent experiments. * $p < 0.05$ compared to $t=0$.

