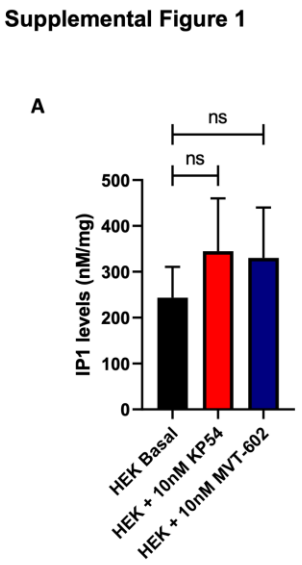
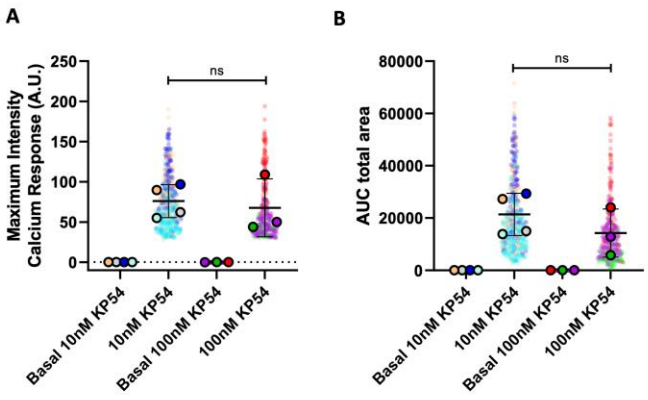
Supplemental Figure 1



Supplemental Figure 1- KP54 and MVT-602 do not increase intracellular levels of IP_1 in HEK 293 cells not expressing KISS1R

HEK 293 denotes cells that were subjected to the transfection process in the absence of DNA for KISS1R. Experiments were conducted 72 h post transfection. Cells were then untreated (basal) or treated with either KP54 or MVT-602 (10 nM) and intracellular levels of IP_1 were measured after a 2 h stimulation. n = 4 independent experiments and data is normalized to protein concentration as described in methods. No significant difference was observed following one-way ANOVA with Tukey's post-test.

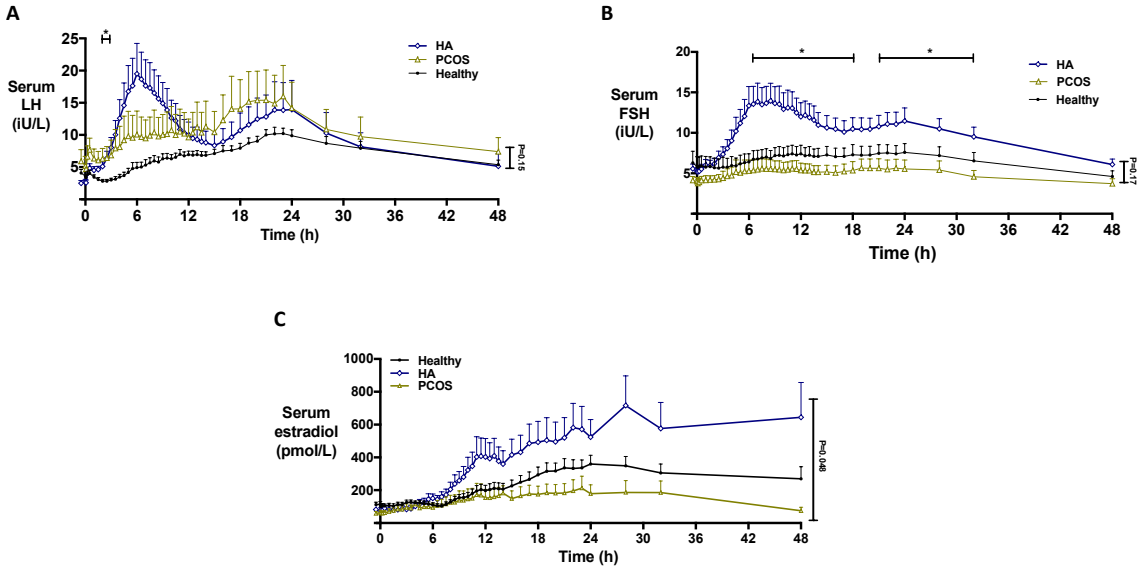
Supplemental Figure 2



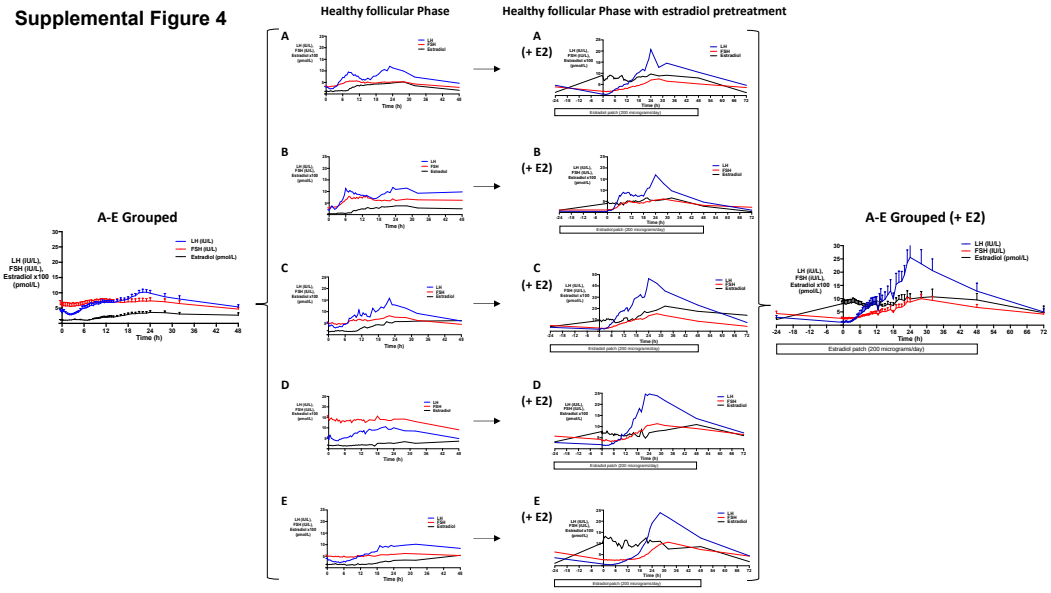
Supplemental Figure 2– Comparison of 10 nM and 100 nM KP54 treatment in acute calcium response in HEK 293 cells

(A): Maximum intensity and (**B):** total area under the curve (AUC), calculated from data following 10 nM or 100 nM KP54 treatment (10 min). Data shows individual cell distribution (total number of cells; 10 nM KP54 n = 547, 100 nM KP54 n = 325) overlaid with the mean (±SD) values of at least n = 3 independent experiment conducted in duplicate wells. Cells attributed to each biological repeat are shown in the corresponding color. There were no significant differences between 10 nM KP54 and 100 nM KP54 following comparison by two-way ANOVA.

Supplemental Figure 3



Supplemental Figure 3 (A-C): Mean (±SEM) of serum LH (iU/L) (A), serum FSH (iU/L) (B) and serum estradiol (pmol/L) (C) in healthy women during the early follicular phase in black (n = 9), women with polycystic ovary syndrome (PCOS) in olive green (n = 6), and women with hypothalamic amenorrhea (HA) in dark blue (n=6) after receiving a single subcutaneous (SC) bolus of MVT-602 (0.03 nmol/kg) at T = 0 h. Mean LH at T= 0 h was: Healthy women 3.6 iU/L, HA 2.6 iU/L, PCOS 5.2 iU/L. Mean FSH at T = 0 h was: Healthy women 5.8 iU/L, HA 5.2 iU/L, PCOS 4.0 iU/L. Mean estradiol level at T = 0 h was: Healthy women 117.3 pmol/L, HA 77.0 pmol/L, PCOS 86.2 pmol/L. Groups were compared by two-way ANOVA (presented vertically on right hand edge of each graph) with post-hoc Tukey's multiple comparison test (presented horizontally across the top of each graph). * indicates P <0.05.



Supplemental Figure 4: LH (iU/L) in blue, FSH (iU/L) in red and estradiol levels (x100 pmol/L) in black over time in five healthy women (**Supplemental Figures 4 A-E**) following a subcutaneous bolus of MVT-602 (0.03 nmol/kg) at T = 0 h in the early follicular phase (on the left), and in (**Supplemental Figures 4 A-E +E2**) in a separate menstrual cycle with estradiol pre-treatment (on the right) at a dose of 200 mcg per day via a transdermal patch starting from 24 h prior to administration of MVT-602 and continued until 48 h after administration of MVT-602. Serum estradiol levels at T = 0 h were **A** 119 pmol/L, **B** 64 pmol/L, **C** 177 pmol/L, **D** 174 pmol/L, **E** 167 pmol/L in unsupplemented studies. Serum estradiol levels at T = 0 h were **A (+E2)** 835 pmol/L, **B (+E2)** 440 pmol/L, **C (+E2)** 951 pmol/L, **D (+E2)** 696 pmol/L, **E (+E2)** 1183 pmol/L in estradiol patch pretreated studies. Grouped data presenting Mean ±SEM for unsupplemented (**A-E grouped**) and estradiol pretreated studies (**A-E grouped + E2**) are also presented.

Supplemental file Video S1 KP54-mediated increase in intracellular Ca^{2+}.

Representative video of HEK 293 cells expressing human KISS1R following incubation with Ca^{2+} indicator dye, Fluo4-AM for 1 h. Cells were imaged live via confocal microscopy for 1 min prior to addition of KP54. Cells were then imaged for a further 60 min. Images were taken every 1.29 seconds and time stamp indicates the initial addition of ligand. Videos play at 10 frames per second.

Supplemental file Video S2 MVT-602-mediated increase in intracellular Ca^{2+}.

Representative video of HEK 293 cells expressing human KISS1R following incubation with Ca^{2+} indicator dye, Fluo4-AM for 1 h. Cells were imaged live via confocal microscopy for 1 min prior to addition of MVT-602. Cells were then imaged for a further 60 min. Images were taken every 1.29 seconds and time stamp indicates the initial addition of ligand. Videos play at 10 frames per second.

Supplementary Methods

Preliminary dose-finding study for MVT-602 in healthy women: As MVT-602 had not been previously administered to women, we conducted a preliminary broad dose-finding study to examine the hormonal profile following administration of MVT-602 in the early follicular phase (menstrual cycle days 1-4) of healthy ovulatory woman (n = 3). Women received a SC bolus of MVT-602 at the following doses: 0.003 nmol/kg, 0.03 nmol/kg, 0.1 nmol/kg, 0.3 nmol/kg and 1.0 nmol/kg in sequential order during the early follicular phase of successive menstrual cycles (with a single dose of MVT-602 administered in each menstrual cycle). These doses of MVT-602 were selected based on a previous phase 2 study conducted in healthy male volunteers (23). Serum reproductive hormone levels (LH, FSH, estradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 14 h, and additionally at 24, 28, 32 and 48 h.

Hormone Assays: Blood samples for serum LH, FSH, estradiol and progesterone measurement were collected in simple Vacutainer tubes (Becton Dickinson, Franklin Labs, New Jersey). Samples were allowed to clot prior to centrifugation for 10 min at 1210 g and then separated. Blood samples for plasma kisspeptin analysis were collected in lithium-heparin tubes (Becton Dickinson, Franklin Labs, New Jersey) containing 5000 Kallikreiin inhibitor units of aprotinin (0.2 ml Trysalol; Bayer, Newbury, UK). Samples were immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) for 4 min at 2150 g separated and stored at -20ºC. Blood for MVT-602 levels was collected in chilled K2EDTA tubes (Becton Dickinson, Franklin Labs, NJ) and was spun immediately for 10 min at 194 g at 4 ºC and stored at -20 ºC until analysis.

Samples were defrosted and analyzed for measurement of serum LH, FSH and estradiol using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Reference ranges were as follows: LH (iU/L), 2-10 (follicular), 20-60 (mid cycle), 4-14 (luteal); FSH (iU/L), 1.5-8.0 (follicular and luteal), 10-50 (mid cycle) and estradiol (pmol/L), less than 300 (early follicular), 400-1500 (mid cycle), 200-1000 (luteal). Interassay coefficients of variation were as follows: LH, 2.7%; FSH, 3.0%; oestradiol, 3.0%; progesterone, 2.9%. Limits of detection for each assay were as follows: estradiol, 37 pmol/L; FSH, 0.05 iU/L; LH 0.05 iU/L; progesterone, 0.32 nmol/L.

Measurement of plasma kisspeptin immunoreactivity (IR) was carried out using an established RIA (8).

MVT 602 levels were measured using liquid chromatography with tandem mass spectrometry by PPD laboratories in Virginia, USA. Measurement of MVT-602 levels were conducted as follows: a 500-µL matrix aliquot was fortified with 25 µL of 0.200 ng/mL internal standard working solution. Analytes were isolated through solid phase extraction using Waters Oasis WCX 30-mg, 96-well SPE plates and eluted with 700 µL of 98:2 methanol:formic acid, v:v. Sample extraction steps were controlled and automated using a MicroLab NIMBUS 96. The eluate was evaporated under a nitrogen stream at approximately 25 °C, and the residue reconstituted with 200 µL of 40:60:0.2 methanol:water:formic acid, v:v:v. The final extract was analyzed via HPLC with MS/MS detection. The limits of detection were 0.5-1000 pg/ml and the coefficient of variation were 4.74-10.45% for QCs and 1.93-14.04% for calibration standards.

Pharmacodynamic (PD) model to characterize LH secretion

The model has the following mathematical form:

$$X(t) - X(0) = P\left(t; t_1, v_{max,1}, \tau_{r,1}, \tau_d\right) + P\left(t; t_2, v_{max,2}, \tau_{r,2}, \tau_d\right),$$

where the function $P(t;\ t_0, v_{max}, \tau_1, \tau_2) = \begin{cases} 0, t < t_0 \\ \dfrac{v_{max}\left(e^{-\frac{t-t_0}{\tau_1}} - e^{-\frac{t-t_0}{\tau_2}}\right)}{\left(e^{-\frac{T_{peak}-t_0}{\tau_1}} - e^{-\frac{T_{peakt}-t_0}{\tau_2}}\right)}, t > t_0, \end{cases}$ with $T_{peak} = t_0 + \frac{\tau_1\tau_2}{\tau_1-\tau_2}\log\left(\frac{\tau_1}{\tau_2}\right)$ describes a single episode in terms of four parameters: t_0 (starting time), v_{max} (maximum increase), τ_1, τ_2 (timescales of rise and decay). The model above was fitted to LH profiles obtained after administration of KP54 and MVT-602. We constrained the first episode to occur between 0 and 10hrs ($0 < t_1 \leq 10$), while the second episode after 10hrs ($t_2 > 10$). Parameter estimates were obtained using maximum likelihood estimation (MLE), that maximizes the likelihood function under the assumption that at each time point the data is distributed according to a Gaussian distribution $N(\mu(t), \sigma(t))$, with mean predicted by the model, i.e., $\mu(t) = X(t)$, and standard deviation obtained from the CV of the assay, i.e., $\sigma(t) = CV \cdot \mu(t)$. Optimization of the likelihood function was performed in MATLAB using genetic algorithm (function ga with population size 10^4).

Effect of MVT-602 and KP54 on human kisspeptin receptor (KISS1R) signaling

Reagents and DNA constructs: FLAG-tagged human KISS1R/pcDNA3 plasmid was kindly provided by Andy V. Babwah (Rutgers University, New Jersey). IP_1 accumulation was measured using an IP-One Gq homogeneous time resolved fluorescence (HTRF) kit (Cisbio, Perkin Elmer) and Ca^{2+} mobilization was conducted using the Fluo-4AM direct Ca^{2+} assay kit (ThermoFisher).

Cell maintenance and transfection: All experiments were conducted in human embryonic kidney cells (HEK 293; mycoplasma free and used at a passage range of between 20 and 40) and were maintained in Dulbeco's Modified Eagles Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) at 37˚C in 5 % CO2, 95% atmospheric air. Cells were

transiently transfected at 60-70% confluency with FLAG-tagged KISS1R at the indicated concentrations, 72 h prior to experimentation. Transfections were performed using Lipofectamine 2000 (ThermoFisher) in Opti-MEM (GIBCO).

Electrophysiological Recordings in GnRH neurons

Animals: Female C57Bl6 mice (GnRH hip mice bread in-house since 1990's) expressing green fluorescent protein (GFP) under the control of the GnRH promoter (JAX 033639) were used (62). Mice were aged 66 to 126 days. Animals were housed on a 14-h light, 10-h dark cycle with lights on at 0400 am Eastern Standard Time. Animals were provided with water and Teklad 2916 chow (Envigo, Madison, WI) *ad libitum*. Vaginal lavage was used to evaluate cytology and all mice were in the diestrus phase of the cycle when brain slices were prepared for recording. All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (PRO00008797).

Brain slice preparation: All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Solutions were bubbled with 95% O_2 and 5% CO_2 throughout the experiments and for at least 15 minutes before exposure to tissues. The brain was removed rapidly and placed in ice-cold sucrose-saline solution containing 250 mM sucrose, 3.5 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.25 mM Na_2HPO_4, 1.2 mM $MgSO_4$, and 3.8 mM $MgCl_2$. Coronal brain slices (300 μm) were prepared with a Leica VT1200S (Leica Biosystems, Buffalo Grove, IL). Slices were incubated in a 1:1 mixture solution of sucrose-saline and artificial cerebrospinal fluid (ACSF) containing 135 mM NaCl, 3.5 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.25 mM Na_2HPO_4, 1.2 mM $MgSO_4$, and 2.5 mM $CaCl_2$ for 30 minutes at room temperature and then transferred to 100% ACSF for at least 30 minutes at room temperature before recording. Slices were placed in the chamber continuously perfused with oxygenated ACSF at a rate of 3 mL/min and heated by an in-line heater (Warner Instruments, Hamden, CT) to maintain temperature at 30°C ± 1°C. GFP-labeled GnRH neurons were identified by brief fluorescent illumination at 488 nm on an upright Olympus BX51W1 fluorescence microscope (Opelco, Dulles, VA).

Electrophysiological recordings: Recording pipettes were pulled from borosilicate capillary glass (type 7052, 1.65-mm outer diameter and 1.12-mm inner diameter; World Precision Instruments, Inc., Sarasota, FL) using a Flaming/Brown P-97 (Sutter Instrument, Novato, CA) to obtain pipettes with a resistance of 2 to 3.5 MΩ when filled with HEPES-buffered solution, containing 150 mM NaCl, 3.5 mM KCl, 10 mM HEPES, 10 mM D-glucose, 1.3 mM $MgCl_2$, and 2.5 mM $CaCl_2$. Targeted single-cell extracellular recordings were performed with an EPC-10 dual-patch clamp amplifier (HEKA Elektronik, Holliston, MA) and Patchmaster software (HEKA Elektronik) as data acquisition software. This method does not alter the intracellular milieu of the cell and minimizes interaction between the recording pipette and cell membrane (63,64). Low resistance seals were made between recording pipette and neuron. Seal resistance was checked every 10 min during the recording and data excluded if the resistance was >30 MΩ. Recordings were made in voltage-clamp mode at 0 mV holding potential, sampled at 20 kHz and filtered at 10 kHz.

Statistical analysis of electrophysiological studies: Data are reported as median ± interquartile range (IQR). Statistical analyses were performed using GraphPad Prism 8. Data were examined for distribution using Shapiro-Wilk. Data during the control period were not normally distributed, attributable to low firing rate on diestrus. Two-way repeated-measures mixed-effects model (restricted maximum likelihood, REML) was used to compare treatment effect on firing rate over time to account for different duration recordings, which resulted in missing values towards the end of some recordings; all three treatments were included in the same analysis. Bonferroni *post hoc* was selected as it is sufficiently robust for non-normally distributed data. Two-tailed Mann Whitney U test was used to evaluate average peak firing rate achieved by each treatment. Significance was set at $P < 0.05$, but exact P-values ≤ 0.1 are reported.