Endogenous Kisspeptin Tone Is a Critical Excitatory Component of Spontaneous GnRH Activity and the GnRH Response to NPY and CART

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Abstract

Background/Aims: Kisspeptin is the major excitatory regulator of gonadotropin-releasing hormone (GnRH) neurons and is responsible for basal GnRH/LH release and the GnRH/LH surge. Although it is widely assumed, based on mutations in kisspeptin and Kiss1R, that kisspeptin acts to sustain basal GnRH neuronal activity, there have been no studies to investigate whether endogenous basal kisspeptin tone plays a direct role in basal spontaneous GnRH neuronal excitability. It is also of interest to examine possible interactions between endogenous kisspeptin tone and other neuropeptides that have direct effects on GnRH neurons, such as neuropeptide Y (NPY) or cocaine- and amphetamine-regulated transcript (CART), since the activity of all these neuropeptides changes during states of negative energy balance. *Methods:* Loose cell-attached and whole-cell current patch-clamp recordings were made from GnRH-GFP neurons in hypothalamic slices from female and male rats. *Results:* Kisspeptin activated GnRH neurons in a concentration-dependent manner with an EC_{50} of 3.32 ± 0.02 nM. Surprisingly, a kisspeptin antagonist, Peptide 347, suppressed spontaneous activity in GnRH neurons, demonstrating the essential nature of the endogenous kisspeptin tone. Furthermore, inhibition of endogenous kisspeptin tone blocked the direct activation of GnRH cells that occurs in response to antagonism of NPY Y5 receptor or by CART.*Conclusions:* Our electrophysiology studies suggest that basal endogenous kisspeptin tone is not only essential for spontaneous GnRH neuronal firing, but it is also required for the net excitatory effects of other neuropeptides, such as CART or NPY antagonism, on GnRH neurons. Therefore, endogenous kisspeptin tone could serve as the linchpin in GnRH activation or inhibition.

Keywords: Electrophysiology , Cocaine- and amphetamine-regulated transcript , Neuropeptide Y, Kisspeptin , Gonadotropin-releasing hormone

Introduction

Gonadotropin-releasing hormone (GnRH) neurons are the major regulators of reproductive function, and they are modulated by sex steroid feedback, metabolic status, stress and various other factors [1,2,3,4]. In turn, the neuropeptide, kisspeptin, encoded by the *Kiss1* gene, is the primary upstream regulator of GnRH neurons through actions on G_q-coupled Kiss1R receptors on the cell membrane [5,6,7]. Mutations of the *Kiss1* gene or Kiss1R result in failure to reach puberty and in infertility in humans and in some, but not all, rodent models [6,7,8,9,10]. Kisspeptin plays a major regulatory role of GnRH, in both pulsatile and surge modes of secretion [11,12,13,14], and antagonism of kisspeptin's actions in adult animals results in a suppression of pulsatile LH secretion and the ovulatory LH surge [15,16,17]. Together these data suggest that the kisspeptin system plays a critical role in regulating GnRH and reproductive function.

The emerging view of kisspeptin signaling in the rodent is that it is responsible for the two modes of GnRH secretion: the arcuate nucleus (ARH) kisspeptin population regulates steroid-negative feedback and basal pulsatile GnRH/LH release through actions at GnRH terminals, whereas the anteroventral periventricular (AVPV) kisspeptin population drives the estrogen-induced ovulatory GnRH surge through direct actions at the GnRH cell body [1,18,19]. Although it is widely assumed, based on mutations in kisspeptin and Kiss1R [6,7,8,9], that kisspeptin acts to sustain basal GnRH neuronal activity, there have been no studies to investigate whether endogenous basal kisspeptin tone plays a direct role in basal spontaneous GnRH neuronal excitability. If such an action of kisspeptin could be demonstrated, endogenous kisspeptin tone could play a critical role in determining GnRH excitability or inhibition during states of negative energy balance when kisspeptin and GnRH are inhibited [1,11,20].

In addition to the essential role of kisspeptin in the regulation of GnRH, many other neuropeptides have been shown to have direct effects on GnRH cells through expression of specific receptors, such as those for the majority of the neuropeptide systems regulating appetite [11,21]. For example, neuropeptide Y (NPY) has direct inhibitory effects on GnRH [22], whereas cocaine- and amphetamine-regulated transcript (CART) has direct excitatory effects on GnRH activity [23]. In addition, the activity of these appetite-regulating neuropeptides changes greatly during states of negative energy balance where NPY activity is greatly increased [11] and CART activity is decreased [23]. Therefore, it is of interest to examine the possible interactions between kisspeptin and neuropeptides, such as NPY and CART, on GnRH neurons.

In the present study we utilized a transgenic GnRH-GFP expressing rat and electrophysiological techniques to: (1) characterize the effects of a kisspeptin agonist and antagonist on GnRH activity in the rat, (2) examine the role of endogenous kisspeptin tone in spontaneous GnRH activity in female and male rats, and (3) determine if there is an interaction between endogenous kisspeptin tone and the direct actions of NPY or CART on GnRH neurons.

Materials and Methods

Animals

All animal experiments were performed in accordance with the National Institutes of Health guidelines for care and use of laboratory animals and approved by the Oregon Health & Sciences University Institutional Animal Care and Use Committee. Adult transgenic rats (Wistar) expressing the enhanced green fluorescent protein (EGFP) under the control of the

GnRH promoter were used in these studies (for details on the rats, see Xu et al. [22]). Animals were housed under standard laboratory conditions with free access to food and water. Adult female rats were ovariectomized and subcutaneously implanted with silastic implants (1 cm/100 g body weight) containing 30 µg/ml estradiol in oil. These capsules create low diestrous levels of serum estradiol (5-15 pg/ml [20,24]) and provide uniform estradiol exposure to mimic basal GnRH/LH secretion, thus excluding any possible positive feedback effects of estradiol that could influence the results. Studies were also conducted on intact adult males, in which there would be basal GnRH/LH secretion.

Brain Slice Preparation

Rats were anesthetized and transcardially perfused with ice-cold oxygenated (95% O_2/5% CO_2) cutting solution (composed of (in mM) 208 sucrose, 2 KCl, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$, 1 $CaCl_2$, 10 HEPES and 10 glucose, adjusted to pH 7.4 with NaOH and continuously aerated). Brains were quickly dissected and 350-µm coronal slices through the preoptic area of the hypothalamus were cut using a vibratome. Hypothalamic slices were incubated at 34°C for 10 min in warm oxygenated artificial cerebrospinal fluid (aCSF) and then stored at room temperature until used for recording. All experiments were performed at room temperature and completed within 4-5 h of obtaining the brain slice to ensure cell viability. A single brain slice was transferred to the recording chamber and constantly perfused with aCSF at the rate of 2-3 ml/min. aCSF consisted of (in mM) 124 NaCl, 5 KCl, 2 $MgCl_2$, 2.6 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, 10 HEPES and 10 glucose, adjusted to pH 7.4 with NaOH.

Electrophysiology

Extracellular loose-patch andwhole-cell patch-clamp recordings were made from the soma of EGFP-GnRH neurons. Neurons were identified using a Carl Zeiss Axioskop 2 FS (Jena, Germany) fitted with epifluorescence and infrared-differential contrast video upright microscopy. Patch pipettes were pulled from borosilicate glass capillaries with inner filaments (World Precision Instruments, Sarasota, Fla., USA) using a pp-830 electrode puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and had resistances of 2-4 MΩ when filled with internal pipette solution (in mM): 128 K gluconate, 2 KCl, 1 $MgCl_2$, 2 MgATP, 0.25 NaGTP, 11 EGTA and 10 HEPES, adjusted to pH 7.4 with NaOH. Electrophysiological experiments were performed using an Axopatch 200B amplifier, and signals were digitized with Digidata 1322A (Molecular Devices, Sunnyvale, Calif., USA) interfaced to a Dell computer. Data was collected using pCLAMP10 software (Molecular Devices) at a sample frequency of 20 kHz, with low-pass filtering at 2 kHz.

GnRH neurons were recorded from slices representing the rostral to caudal extent of GnRH neuronal expression. GnRH neurons chosen for recording had intact morphology and were within 50 µm from the slice surface. Adequate whole-cell access (Ra <20 MΩ) and membrane resistance (>500 MΩ) were verified at the beginning and at the end of recording and were electronically compensated. Resistance was monitored throughout the recording and neurons with more than a 20% change in series resistance were rejected. Extracellular loose-patch recording was used to determine the basal spontaneous GnRH neuronal activity in hypothalamic slices from female and male rats. The effects of activation and inhibition of GnRH neurons were determined by analysis of spontaneous action potential firing in loose-patch recordings under voltage-clamp conditions. Drug effects on membrane potential were measured under whole-cell current-clamp conditions in the presence of TTX (1 µM, tetrodotoxin citrate; Tocris Bioscience, Bristol, UK) to block activation of sodium channels. The EC_{50} for the kisspeptin agonist (Kp-10, Mouse KiSS-1 (110-119)-NH_2; Phoenix Pharmaceuticals, Belmont, Calif., USA) and of the effective concentration of the kisspeptin

antagonist, Peptide 347, were determined using hypothalamic slices from female rats and measuring changes in the membrane potential under whole-cell current-clamp conditions in the presence of TTX. The effects of the NPY Y5 receptor (Y5R) antagonist (300 nM) or CART (30 nM) on GnRH neurons were also determined by similar analysis of spontaneous action potential firing and membrane potential change. The concentrations of the Y5 antagonist and CART are based on previous studies from our group [22,23].

To further establish the specificity of Peptide 347, current-voltage (I-V) relationships were generated under no presynaptic block conditions to demonstrate that the kisspeptin-induced current was inhibited by Peptide 347. We used a maximum concentration of kisspeptin (100 nM) to induce consistent and quick depolarization in all cells. For similar reasons we also used a high concentration of Peptide 347 (30 nM). Cells were observed under current clamp for the effects of kisspeptin and Peptide 347 and allowed to reach plateau before I-V plots were constructed. Kisspeptin-induced currents were measured at a holding potential of -55 mV. Since the membrane potential varied from -51 to -66 mV in the GnRH neurons examined in this study, -55 mV was chosen as it was closer to the more depolarized end of the range. Steady-state I-V plots were constructed with step command potentials from -30 to -120 mV with steps of 10 mV and durations of 1 s. A similar protocol was followed to identify the nature of current induced by the Y5 antagonist under voltage clamp. All membrane potentials were corrected for liquid junction potential by -10 mV for final analysis.

The drugs used in the preparation of solutions were from Sigma-Aldrich (St. Louis, Mo., USA) unless otherwise noted. All drug stocks were diluted in aCSF and delivered into the perfusion solution. The ion channel blockers/activators used were CART (55-102, Rat) (American Peptide Co.,Sunnyvale [,]Calif. [,]USA) and Y5 antagonist, L152,804, Tocris Bioscience, UK.

Data Analysis and Statistics

Electrophysiological recordings were analyzed with Clampfit-10 software (Molecular Devices). For event analysis of individual spontaneous firing in loose-patch and action potential firing under current-clamp conditions, Mini Analysis software (Synaptosoft, Inc., Fort Lee, N.J., USA) was used. For firing analysis, the control period was the 3-min interval before drug application, and the drug effect was analyzed during a 3-min interval, starting 5 min after drug treatment. To calculate the change in frequency, events were divided by bin size (time) before and after treatment. Paired t test was used to compare spontaneous firing and action potential firing percentage of GnRH neurons before and after drug treatment, $p = 0.05$ for significance. For membrane potential analysis, the same time intervals pre- and post-drug were used as described above for firing analysis. Statistical evaluation of mean differences in membrane potential after different treatments was performed by one-way ANOVA, with a significance at $p = 0.05$. GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif., USA) was used for these analyses. The EC_{50} for Kp-10 and the ED for Peptide 347 were calculated from the logistic equation derived from the mean data points at each concentration. All data are expressed as mean ± SEM.

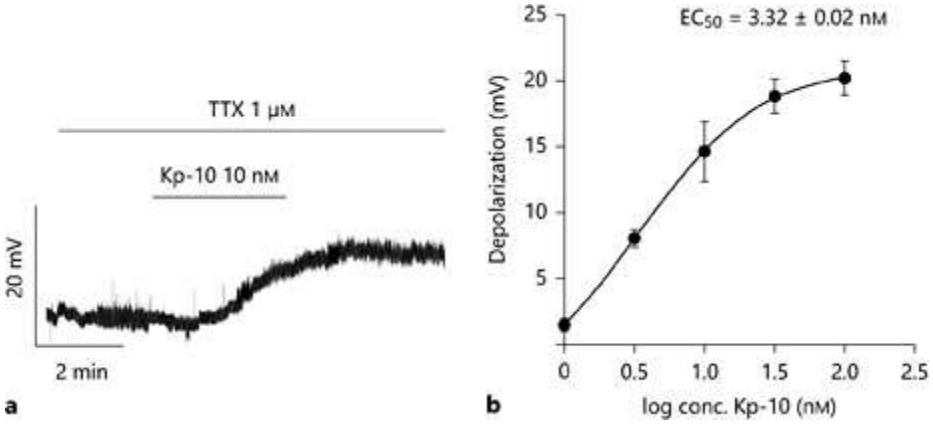
Results

Kisspeptin Depolarizes GnRH Neurons in the Rat in a Concentration-Dependent Manner

Kisspeptin has been shown to have a very prolonged postsynaptic depolarizing effect on GnRH neurons in a concentration-dependent manner in mice [25,26,27]. Here we demonstrate a similar response exists in our experimental model of GnRH-GFP female rats in which membrane potentials were measured under whole-cell current-clamp conditions and in the presence of TTX (1.0 μM). The mean resting membrane potential of GnRH neurons was -

60.2 ± 1.3 mV (n = 28 cells from 16 animals). The kisspeptin agonist, Kp-10, induced a very prolonged, concentration-dependent depolarization of GnRH neurons that persisted long after washout of the agonist (fig. 1a). Approximately 90% of cells responded to Kp-10 (fig. 1a, b). The EC_{50} for the Kp-10-induced depolarization was 3.32 ± 0.02 nM (fig. 1b, n = 6-12 cells from 3-5 animals for each concentration). The maximum depolarization evoked by 100 nM Kp-10 was 20.2 ± 1.2 mV. We chose to use a submaximal dose of 10 nM Kp-10 in subsequent studies.

Fig.1 : Kisspeptin (Kp-10) depolarizes GnRH neurons from female rats in a concentration-dependent manner. a Representative current-clamp recording in the presence of TTX showing depolarization by 10 nM Kp-10. Resting membrane potential was -62 mV. b Dose-response curve for the kisspeptin-induced depolarization. The EC_{50} for the kisspeptin-induced depolarization was 3.32 ± 0.02 nM (n = 6-12 cells from 3-5 animals for each concentration; 1, 3, 10 and 100 nM). Data are presented as mean ± SEM. The EC_{50} for Kp-10 was calculated from the logistic equation derived from the mean data points at each concentration.



Endogenous Basal Kisspeptin Tone in Female Rats

Based on mutations in kisspeptin and Kiss1R [6,7,8,9], it has been widely assumed that kisspeptin acts to sustain basal GnRH neuronal activity, although there is no direct evidence for this role of kisspeptin. To address this question, we utilized a novel kisspeptin antagonist, Peptide 347, to demonstrate the endogenous kisspeptin tone. The kisspeptin antagonist, Peptide 347, was derived from Peptide 234, a peptide whose structure has been published [15]. Synthesis was done by conventional solid phase and HPLC purification to yield >80% purity, creating an antagonist with increased stability and longer duration of action. The specificity of Peptide 347 was first examined in vitro using CHO cells transfected with the human Kiss1R (see Supplemental Material for details). The K_d (dissociation constant) for Peptide 347 was 1.0 nM, whereas for Peptide 234 and Kp-10 was 2.7 nM[15]. Also, the ED_{50} for Peptide 347 inhibition of 10 nM Kp-10 stimulation of inositol phosphate in CHO cells was 1.91 nM (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000365419, with methodological details), compared to 7 nM for Peptide 234 [15]. Taken together, these data suggest that Peptide 347 is a specific, high-affinity antagonist to kisspeptin. Additional support for the specificity of the peptides derived from Peptide 234 comes from use of another similarly derived kisspeptin antagonist, Peptide 318, which has recently been reported to inhibit GnRH neuronal firing in response to AVPV-stimulated kisspeptin release [28].

To assess the role of endogenous kisspeptin tone, extracellular loose-patch recordings were made from spontaneously active GnRH neurons in slices from female rats. Peptide 347 (10 nM, a submaximal dose, see fig. 4) suppressed spontaneous firing events from >30 to 1/min by 10-15 min after exposure (fig. 2a, b). It should be noted that there was a 5- to 10-min delay before the effects of the antagonist were observed, and its effects persisted after drug

wash off. The average firing frequency after Peptide 347 exposure was significantly suppressed (fig. 2c: before 7.0 ± 3.3 Hz, after 1.1 ± 0.3 Hz; n = 8 cells from 5 animals; $p < 0.005$). Subsequent recordings used the current-clamp configuration and demonstrated spontaneous action potential events, followed by an almost complete cessation of firing by 7-10 min after addition of Peptide 347 (fig. 2d, e). Subsequent addition of Kp-10 stimulated the cell, indicating its continued viability. Overall, the action potential frequency after Peptide 347 exposure was significantly decreased (fig. 2f: before 2.8 ± 0.9 Hz, after, 0.36 ± 0.1 Hz; n = 10 cells from 5 animals; $p < 0.05$), demonstrating that endogenous kisspeptin tone is a critical component of basal GnRH activity.

Fig. 2: Endogenous kisspeptin tone is a major component of spontaneous GnRH activity in female rats. aRepresentative loose cell-attached recording before and after Peptide 347 exposure. Peptide 347 inhibits spontaneous neuron firing. b Histogram showing number of events/minute for the recording in panel a. The horizontal bar indicates the duration of Peptide 347 administration. cBar graph showing analysis of mean action potential firing frequency of loose-patch recordings of neurons before and after Peptide 347 exposure (n = 8 cells from 5 animals, ** $p < 0.005$).For firing analysis, the control period was the 3-min interval before Peptide 347 application and the drug effect was analyzed during a 3-min interval, starting 5 min after Peptide 347 treatment. dExample of current-clamp recording demonstrating inhibition of spontaneous action potential firing of GnRH neurons by Peptide 347. Resting membrane potential was -63 mV. The effect was reversible with wash off and application of Kp-10. e Histogram showing number of action potential events/minute for the recording in panel d. The horizontal bar indicates the duration of Peptide 347 administration. f Bar graph showing analysis of mean action potential firing frequency of current-clamp recordings of neurons, before and after Peptide 347 exposure (n = 10cells from 5 animals, ** $p < 0.005$). See panel c for time intervals for firing analysis.

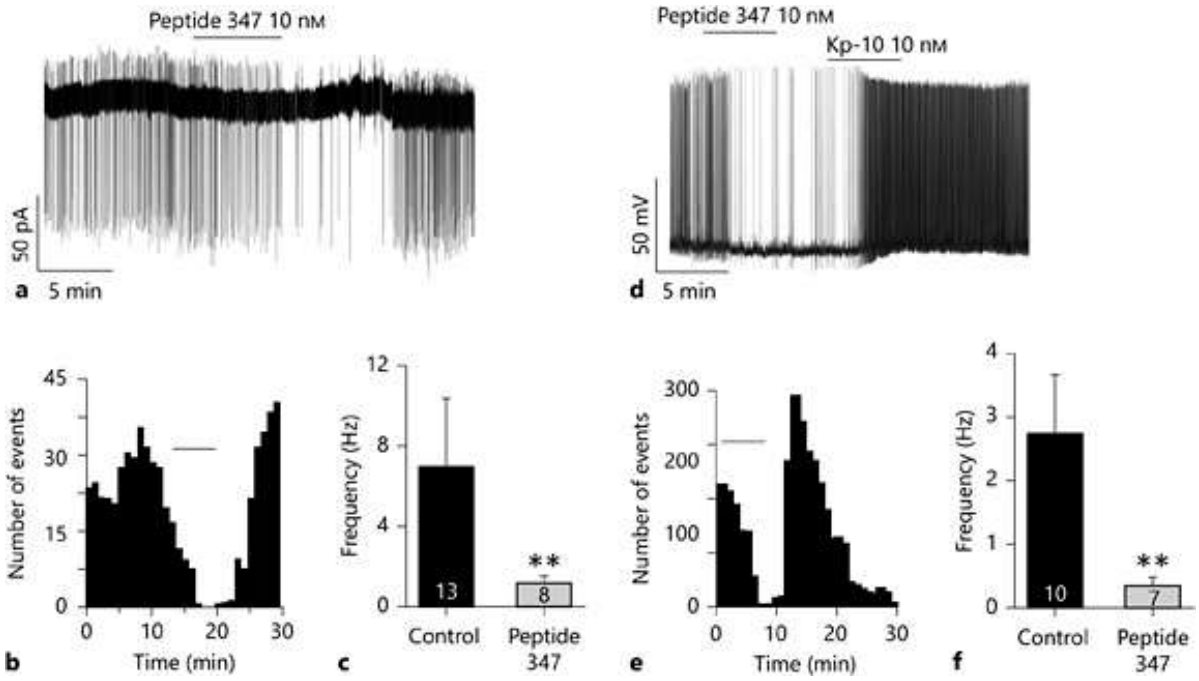
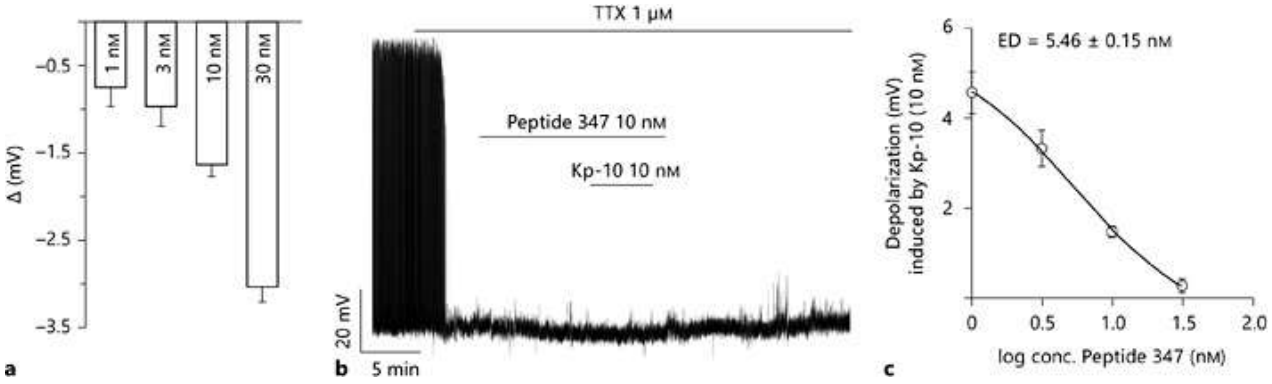


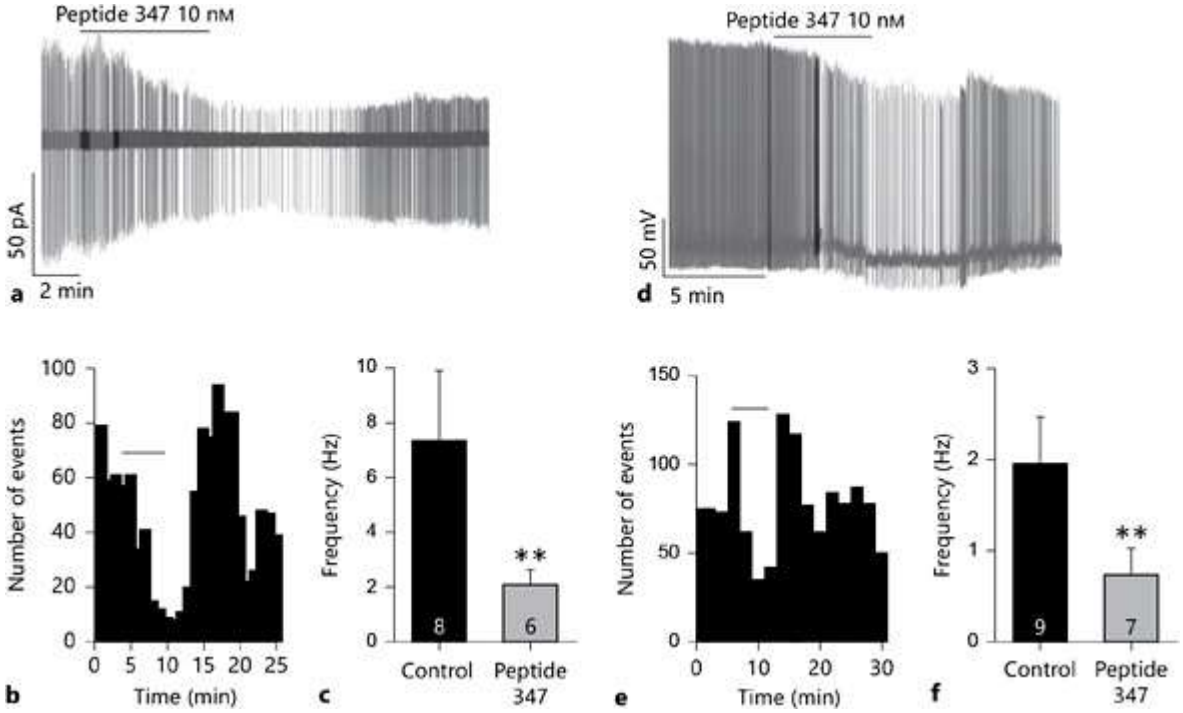
Fig. 4: Kisspeptin antagonist, Peptide 347, inhibits Kp-10-induced depolarization of GnRH neurons from female rats in a concentration-dependent manner. a Bar graph demonstrating average hyperpolarization of GnRH neurons after increasing concentrations of Peptide 347 (under TTX) ($p < 0.0001$). b Example ofcurrent-clamp recording in the presence of TTX showing that Kp-10-induced depolarization is inhibited postsynaptically by Peptide 347. The resting membrane potential was -61.2 mV. c Concentration of Peptide 347 (log inhibitor) versus response curve for the inhibition by Peptide 347 of Kp-10 (10 nM) induced depolarization (effective concentration 5.46 ± 0.15 nM). The effective concentration for Peptide 347 was calculated from the logistic equation derived from the mean data points at each concentration. Data are presented as mean ± SEM. n = 32 cells from 21 animals, 10 nonresponding cells. Note: Because the antagonist was applied first, followed by the 10 nM Kp-10, it was not possible to determine if the cell was responsiveness or nonresponsive (about 10-15% of cells) to kisspeptin. To establish that the cell was a kisspeptin-responsive cell, the cells were washed for 15 min to remove both antagonist and agonist and then Kp-10 was applied again. If the cell responded to this second concentration of kisspeptin, it was deemed a responsive cell. Using this protocol, the time of recording a cell was greatly extended and the number of nonresponders was increased.



Basal Endogenous Kisspeptin Tone in Male Rats

It has been shown that kisspeptin modulates GnRH neuronal secretion in male as well as female rodents [5,29]. GnRH neurons in the male express Kiss1R [5,26,30], as in the female [31], although kisspeptin fiber contacts on GnRH neurons are 3- to 4-fold less in the male than female [32]. Furthermore, kisspeptin has been shown to activate about 90% GnRH neurons in the adult male mouse [26]. Therefore, we assessed whether kisspeptin was necessary to sustain basal GnRH neuronal activity in the male, as was demonstrated for the female. Extracellular loose-cell recordings were made of spontaneously active GnRH neurons in slices from intact male rats. 10 nM Peptide 347 suppressed spontaneous firing events from >50 to close to 10/min after exposure (fig. 3a, b), and the overall average firing frequency was significantly suppressed (fig. 3c: before 7.4 ± 2.5 Hz, after 2.1 ± 0.5 Hz; n = 5 cells from 3 animals; $p < 0.05$). Using the current-clamp configuration, spontaneous action potential events were suppressed from 60 to 13/min after application of Peptide 347 (fig. 3d, e), and there was a significant decrease in the average action potential frequency (fig. 3f: before 2.0 ± 0.5 Hz, after 0.75 ± 0.3 Hz; n = 6 cells from 4 animals; $p < 0.05$). These results demonstrate that endogenous kisspeptin tone is also present in male rats and plays an important role in spontaneous GnRH activity.

Fig.3: Kisspeptin tone contributes to spontaneous GnRH activity in male rats. a Representative loose cell-attached recording before and after Peptide 347 exposure. Peptide 347 greatly reduces spontaneous neuron firing. b Histogram showing number of events/minute for the recording in panel a. The horizontal bar indicates the duration of Peptide 347 administration. c Bar graph showing analysis of mean action potential firing frequency of loose-patch recordings of neurons before and after Peptide 347 exposure (n = 5 cells from 3 animals, ** $p < 0.005$). Time intervals for control and Peptide 347 firing analysis are described in figure 2c. d Example of current-clamp recording demonstrating suppression of spontaneous action potential firing and hyperpolarization of GnRH neurons by Peptide 347. Resting membrane potential was -61.3 mV. eHistogram showing number of action potential events/minute for the recording in panel d. The horizontal bar indicates the duration of Peptide 347 administration. f Bar graph showing analysis of mean action potential firing frequency of current-clamp recordings of neurons before and after Peptide 347 exposure (n = 6 cells from 4 animals, ** $p < 0.005$). Time intervals for control and Peptide 347 firing analysis are described in figure 2c.



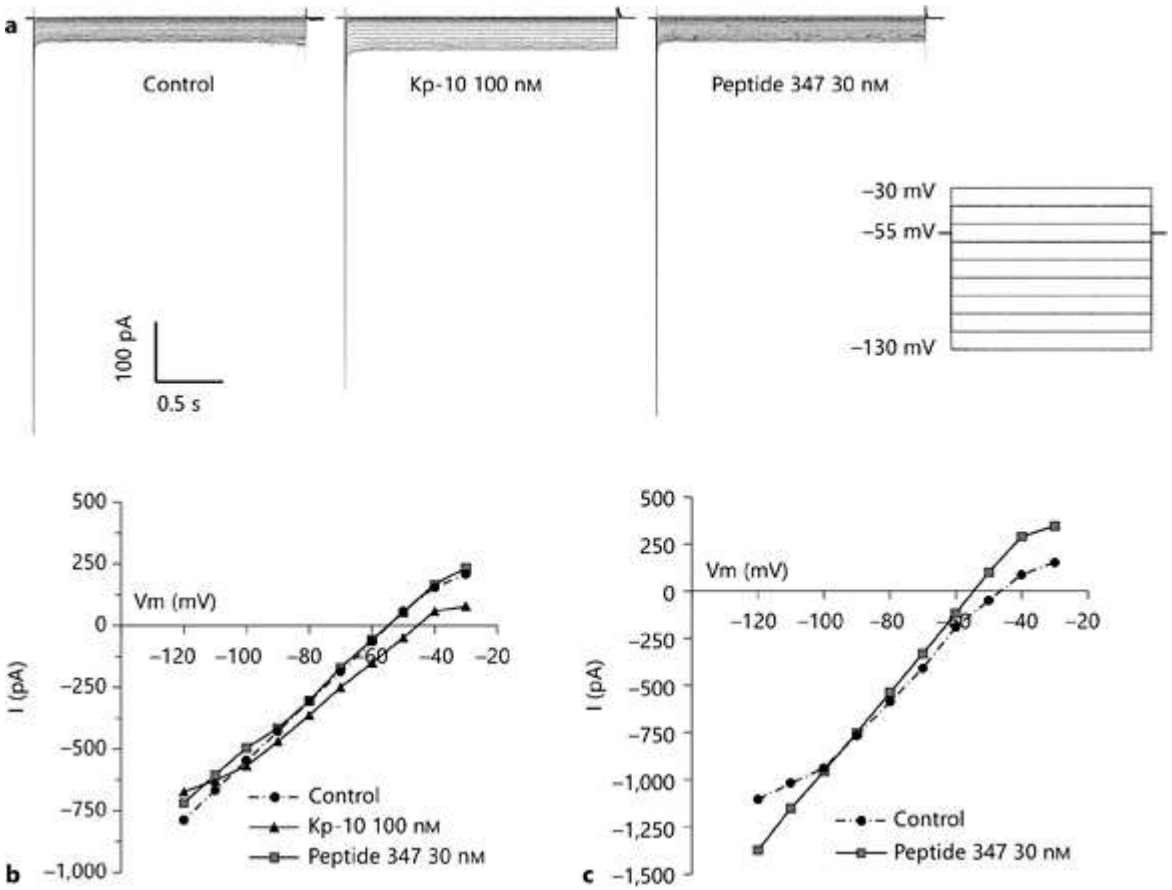
Kisspeptin Antagonist, Peptide 347, Acts Directly on GnRH Neurons to Suppress Kisspeptin-Induced Depolarization in a Concentration-Dependent Manner

To further elucidate the actions of Peptide 347, GnRH neurons in hypothalamic slices from female rats were studied in the presence of TTX to determine the direct, postsynaptic, concentration-dependent effects of the antagonist on the GnRH cell. Application of Peptide 347 produced a small but significant hyperpolarization of the membrane potential (fig. 4a), demonstrating direct actions on the GnRH cell and providing additional support for the presence of an endogenous kisspeptin tone. The concentration-dependent inhibitory effects of Peptide 347 were measured against the constant stimulus of 10 nM Kp-10, a submaximal dose that causes a depolarization of 14.6 ± 2.3 mV (see fig. 1b). Following application of TTX and inhibition of spontaneous action potential firing, application of Peptide 347 prior to application of 10 nM Kp-10 suppressed the Kp-10-induced depolarization to 4.5 ± 1.3 (1 nM), 3.32 ± 0.4 (3 nM), 1.47 ± 0.12 (10 nM) and 0.27 ± 0.15 (30 nM), respectively (fig. 4b, c). The effective concentration of Peptide 347 against the 10 nM Kp-10 stimuli was 5.46 ± 0.15 nM (fig. 4c). Peptide 347 at 10 nM (submaximal dose), and higher concentrations (up to 1 μM) produced consistent inhibitory effects and no agonist effects were observed.

Peptide 347 Reverses the Kisspeptin-Induced Current in GnRH Neurons from Female Rats

To further explore the specificity of Peptide 347 antagonistic effects, I-V curves were generated to determine the ionic nature of the inward currents induced by kisspeptin and their reversal by Peptide 347. A maximum concentration of 100 nM Kp-10 was used to evoke a fast and maximum response. I-V relationships were obtained immediately before Kp-10 application, after Kp-10-induced depolarization reached steady state, and after the cell reached a hyperpolarized plateau following application of 30 nM Peptide 347 (see representative tracings in fig. 5a). The recording in figure 5b shows the kisspeptin-induced current reversed at -110 mV (compared to control). In 65% of the cells (8 out of 12 cells from 5 animals), the kisspeptin-induced current was reversed between -100 and -110 mV after Peptide 347 exposure (fig. 5b). In the other 35% of the cells (4 cells), there was no apparent current reversal. Application of 30 nM Peptide 347 alone induced hyperpolarization (6 out of 8 cells from 4 animals) and the currents reversed between -100 and -105 mV (fig. 5c, representative recording). The similarity in reversal potentials between Kp-10 and Peptide 347 is consistent with Peptide 347 acting at the Kiss1R.

Fig. 5: Peptide 347 suppresses kisspeptin-evoked inward current in GnRH neurons from female rats. aRepresentative traces showing I-V relationship of sequential exposure to control, Kp-10 and Peptide 347 of the same GnRH cell from a female rat. The panel on the right shows the voltage-clamp protocol used. b Typical I-V curve obtained before and after Kp-10 application (E_{rev} = -110 mV). In 65% (8 of 12 cells from 5 animals) the kisspeptin-induced current is reversed between -100 and -110 mV by subsequent Peptide 347 application. c I-V curve before and after Peptide 347 application. The current reversed at -100 mV (compared to control).



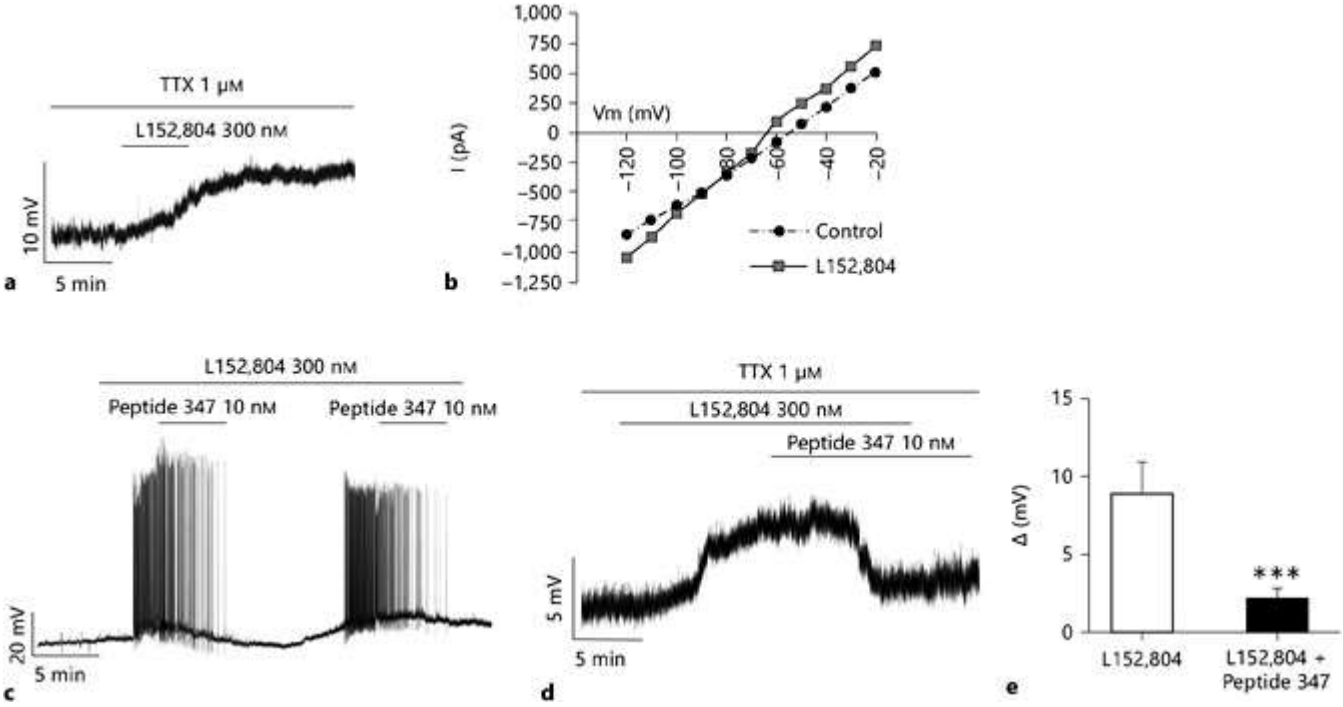
Depolarization of GnRH Neurons by Antagonizing the Y5R Requires Endogenous Kisspeptin Excitatory Tone

Previous work from our laboratory demonstrated that the NPY Y5R postsynaptically inhibits GnRH neuronal firing in female rats, and blocking the Y5R can activate GnRH neurons, demonstrating the presence of an endogenous inhibitory NPY tone [11,22]. However, whether endogenous kisspeptin tone is required for other neuropeptides, such as NPY, to have these effects has not been demonstrated. The possible interaction between Y5R and Kiss1R at the GnRH cell body is of physiological interest, since GnRH neurons are subjected to both endogenous excitatory kisspeptin (see above) and inhibitory NPY tone [22]. Thus, changes in the relative tone of either factor could alter basal GnRH activity.

Whole-cell current-clamp recordings of GnRH neurons from slices of female rats were performed before and after addition of the specific Y5R antagonist (300 nM L152,804) in the presence of TTX to demonstrate the postsynaptic nature of the Y5R on GnRH neurons. Approximately 75% of GnRH neurons exhibited a prolonged depolarization in response to the application of L152,804 (fig. 6a; n = 20 cells from 12 animals), demonstrating the presence of an endogenous inhibitory NPY tone, as reported previously by our group [22]. The average change in membrane potential was 8.9 ± 2.0 mV, which was significantly different when compared to controls ($p < 0.05$). The I-V plot established in response to antagonizing the Y5R (fig. 6b) showed that the L152,804-induced inward current was reversed between -80 and -90 mV (n = 7 cells from 3 animals) which is close to E_{K^+} (-90 mV), suggesting that the Y5R antagonist-induced effect may result from inhibition of GIRK or Kir channels.

To demonstrate a possible interaction between inhibitory NPY and excitatory kisspeptin actions on GnRH neurons, we utilized the kisspeptin antagonist, Peptide 347. Using whole-cell current-clamp, a quiescent GnRH cell was depolarized and action potential firing was induced by the 300 nM L152,804, demonstrating it was a Y5R-positive GnRH cell. This firing was subsequently suppressed by 10 nM Peptide 347 (fig. 6c). Recording in the presence of TTX, to show the postsynaptic nature of this interaction, 300 nM L152,804 depolarized the GnRH cell (fig. 6d, e: 8.9 ± 2.0 mV); this depolarization was significantly attenuated by subsequent application of Peptide 347 (fig. 6d, e: 2.42 ± 1.06 mV; n = 16 cells from 7 animals; $p < 0.0001$). These data suggest that under basal conditions, GnRH neurons can be quiescent when endogenous inhibitory NPY tone predominates over endogenous excitatory kisspeptin tone. However, after removal of the inhibitory NPY tone, endogenous kisspeptin tone is necessary for activation of GnRH neurons.

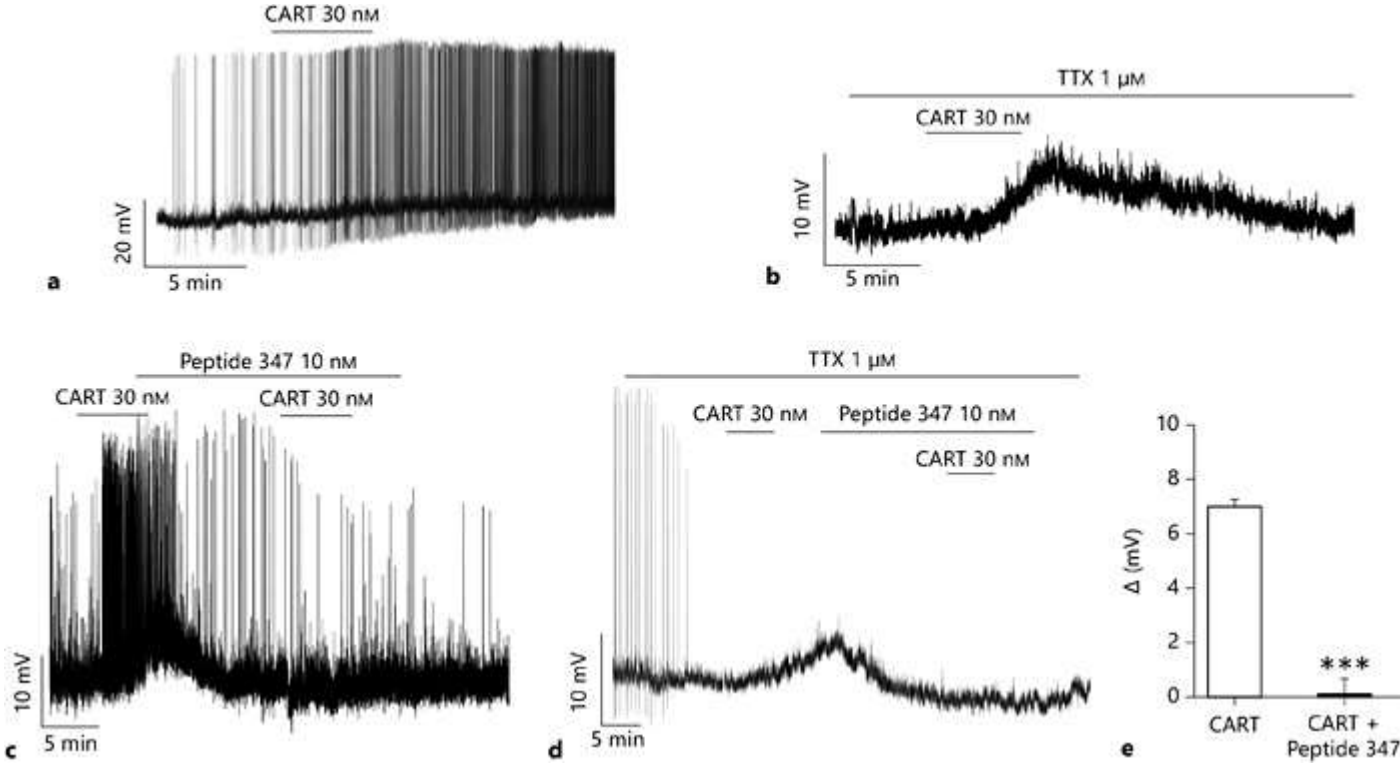
Fig. 6: Peptide 347 suppresses GnRH depolarization induced by the Y5 antagonist, L152,804. a An example of a current-clamp recording in the presence of TTX showing that the Y5 antagonist, L152,804 (300 nM), depolarizes GnRH neurons from female rats. Resting membrane potential was -62 mV. b The mean I-V plot shows that L152,804-induced currents reversed at ≈ -85 mV (n = 7 cells from 3 animals). c Representativecurrent-clamp recording of a quiescent GnRH cell in which the Y5 antagonist, L152,804, induces depolarization and spontaneous AP firing. Resting membrane potential was -60.6 mV. Application of Peptide 347 blocks the AP firing and reverses the depolarization. d Under TTX, L152,804 induces depolarization with no spiking; the depolarization is largely suppressed by Peptide 347. Resting membrane potential was -63.3 mV. eHistogram showing analysis under TTX of depolarization induced by L152,804 alone (8.9 ± 2.0 mV) and in the presence of Peptide 347 (2.17 ± 0.6 mV, n = 16 cells from 7 animals, *** $p < 0.0001$) respectively. For analysis of the change in membrane potential, the control period was the 3-min interval before L152,804 application, and the drug effect was analyzed during a 3-min interval, starting 5 min after L152,804. When the peak depolarization occurred, Peptide 347 was added and membrane potential was analyzed for a 3-min interval starting 10 min after Peptide 347 application.



CART Depolarization of GnRH Neurons Requires Endogenous Kisspeptin Tone

To further confirm the interaction of kisspeptin with neuropeptides on the GnRH neuron, we tested the effects of CART, an excitatory neuropeptide, in the presence or absence of endogenous kisspeptin tone. We have shown that CART depolarizes GnRH neurons in female rats in a concentration-dependent manner, with an EC_{50} of 37.4 ± 1.6 nM[23]. Under whole-cell current-clamp conditions, between 60 and 70% of GnRH neurons (8 out of 12 cells from 5 animals) responded to 30 nM CART with a significant membrane depolarization of 7.8 ± 0.1 mV ($p < 0.0001$) and increased firing (fig. 7a). The effect of CART persisted long after washout, as reported previously [23], an effect that is similar to the long-lasting effects of kisspeptin. In the presence of TTX, 30 nM CART evoked a maximum depolarization of 7.3 ± 0.6 mV (fig. 7b; n = 12 cells from 6 animals), confirming our previous study [23] and suggesting that CART's stimulation of GnRH neurons may be primarily a postsynaptic effect.

Fig.7: Peptide 347 suppresses GnRH depolarization induced by CART. a Example of CART-induced depolarization and spontaneous firing in GnRH neurons from female rats. Resting membrane potential was -62 mV. b Example of CART-induced depolarization, under TTX, with no spiking. Resting membrane potential was -64 mV. c Example ofPeptide 347 suppressing the CART-induced spontaneous firing and depolarization of GnRH neurons. Resting membrane potential was -61 mV. d Under TTX, CART depolarizes GnRH neurons and this effect is suppressed by application of Peptide 347. Resting membrane potential was -62.5 mV. e Histogram summarizing the depolarizing effects of CART under TTX and the suppressing effects of Peptide 347 (CART alone, 7.3 ± 0.64 mV; CART with Peptide 347, 0.66 ± 0.6 mV; 12 cells from 6 animals, *** $p < 0.001$).For the analysis of the change in membrane potential, see details in figure 6e.



Recordings using the whole-cell current-clamp configuration were made to test whether inhibition of kisspeptin tone could alter CART's stimulatory actions on GnRH neurons. CART (30 nM) was first added to establish that the GnRH neuron was a CART-responsive cell (fig. 7c). Following application of 10 nM Peptide 347 (n = 13 cells from 6 animals), the depolarization and action potential firing induced by the second application of 30 nMCART was suppressed fig. 7c). It should be noted that earlier studies demonstrated the ability of GnRH cells to respond to a second application of CART [23]. Since most of the CART effects appear to be postsynaptic in nature (fig. 7b [23]), the effects of Peptide 347 on CART depolarization were examined in the presence of TTX. Following TTX-induced suppression of spontaneous action potentials, CART-induced depolarization was countered by Peptide 347 (fig. 7d), and the average change in membrane potential was significantly attenuated following application of Peptide 347 (fig. 7e: CART alone, 7.3 ± 0.64 mV, CART + Peptide 347, 0.01 ± 0.7 mV; n = 12 cells from 6 animals; $p < 0.0001$). Suppression of CART-induced depolarization by antagonizing endogenous kisspeptin tone provides further evidence that kisspeptin tone is a critical component of excitatory responses (CART or antagonizing Y5R) on GnRH neurons.

Discussion

The importance of the direct excitatory effect of kisspeptin on GnRH neurons is well documented. Here for the first time, our electrophysiology studies using hypothalamic slices provide evidence that basal endogenous kisspeptin tone appears to be essential for spontaneous GnRH neuronal firing in female and male rats, since antagonism of endogenous kisspeptin activity suppresses spontaneous GnRH activity. We also found that the direct actions of NPY and CART on GnRH neurons are greatly dependent on the presence of endogenous kisspeptin tone. Therefore, our results implicate endogenous basal kisspeptin tone as a major factor in determining the final state of excitability of GnRH neurons.

In examining the two distinct populations of kisspeptin neurons in rodents (ARH and AVPV) that modulate GnRH neurons, kisspeptin fiber projection studies in the female suggest that the majority of kisspeptin-immunoreactive fibers around GnRH cell bodies arise from cells in the AVPV and this population has been assumed to be responsible for the GnRH/LH surge [1,29,33]. AVPV kisspeptin neuronal expression is almost 25 times greater in females as compared to adult males [34,35], and projections from the AVPV are more robustly expressed in females than in males [32,36], reflecting the sexually dimorphic nature of the AVPV [37,38]. However, a critical role for endogenous kisspeptin tone on GnRH activity in the male was clearly demonstrated by these studies, reflecting the abundance of kisspeptin fibers in close apposition to GnRH cells in the male and the high level of responsiveness of GnRH neurons to kisspeptin in the male [26,32]. Regardless of the origin of the kisspeptin fibers, AVPV or ARH, our results expand the role of kisspeptin neuronal input to GnRH neurons as also providing critical endogenous basal excitatory tone that is critical for spontaneous GnRH activity and action potential firing in both female and male rats.

Our results in the rat regarding kisspeptin's actions on GnRH neurons are in agreement with previous findings in the mouse. Kisspeptin stimulated GnRH neurons in a concentration-dependent manner, with a similar EC_{50} of 3.32 ± 0.02 nM, as shown in mice [27]. Nearly 90% of the GnRH cells responded to kisspeptin in our experiments; this correlates with the Kiss1R receptor expression on GnRH neurons and with c-*Fos* induction data [5,39]. Kisspeptin acts through the $G_{q/s}$-coupled Kiss1R mediating GnRH activation [40], leading to sustained membrane depolarization and increased action potential firing [27]. Data from mice and guinea pigs have shown that kisspeptin-induced currents in GnRH neurons result from simultaneous inhibition of resting Kir (inwardly rectifying potassium) channels [41] and activation of the canonical transient receptor potential (TRPC) channel to yield a reversal potential (-100 to -110 mV), significantly more negative than E_k+ (-90 mV) [27,42,43]. We found similar I-V relationships in the rat, so it is reasonable to speculate that the kisspeptin-induced currents in GnRH neurons in the rat may also consist of activation of TRPC channels and inhibition of potassium currents as shown by Zhang et al. [27] in the mouse.

We used a novel kisspeptin antagonist Peptide 347, a longer duration, highly potent derivative of Peptide 234, to demonstrate the presence of endogenous kisspeptin tone acting on rat GnRH neurons. We found similar results with Peptide 318 [28], which is another longer duration derivative of Peptide 234, although it is less potent than Peptide 347. Peptide 234 has been shown to inhibit kisspeptin-induced activation of GnRH neurons in both in vitro and in vivo experiments in rodents and primates [15,16,17],although in one experiment, there was no effect of Peptide 234 on spontaneous GnRH activity [15]. There does not appear to be an obvious explanation for the difference in results; however, our studies are much more extensive, and Peptide 347 is a more potent antagonist because of its much lower K_d and ED. Our results from in vitro and brain slice studies provide several lines of evidence that Peptide 347 is acting as a Kiss1R antagonist: (1) Peptide 347 blocked the kisseptin-induced inositol

phosphate production in CHO cells; (2) the effects of Peptide 347 were concentration-dependent in nature, similar to kisspeptin [27]; (3) Peptide 347 reversed the kisspeptin-induced current (-100 to -110 mV), and (4) the negative reversal potential of Peptide 347 alone (-100 to -105 mV) demonstrated the similarity of the currents affected by kisspeptin and Peptide 347 [27]. Importantly, Peptide 347 suppressed neuronal firing in 80-90% of the GnRH cells in hypothalamic slices from both female and male rats, and the effects were similar in the presence or absence of TTX, suggesting that most of the antagonist's effects were postsynaptic in nature, similar to the effects of the Kp-10 agonist.

Although kisspeptin has been shown to modulate other neuropeptide systems - it depolarizes and increases firing in POMC neurons and inhibits activation of orexigenic NPY neurons [44,45] - the potential interaction between kisspeptin and neuropeptides at the GnRH neuron is largely unknown but of physiological importance. We began our exploration with NPY, as previous work from our group and others has demonstrated the following: (1) the inhibitory orexigenic NPY neurons directly innervate approximately 50% of GnRH neurons [46]; (2) the majority of the NPY fibers originate from the ARH [46,47]; (3) the NPY Y5R are expressed on about 50% of GnRH cells in the rat [48], and (4) antagonizing the Y5R blocks the effects of endogenous inhibitory NPY tone, primarily through postsynaptic effects [22], and results in activation of the majority of GnRH neurons, reflecting the percentage of GnRH neurons that express the Y5R [48]. The data from this study confirm our previous findings in the rat [22]. Although recent work done in mice demonstrated a very limited effect of NPY Y5R antagonism on GnRH neurons [49], it is possible there may be substantial species differences when it comes to NPY receptor pharmacology in rats and mice. The NPY Y5R antagonist-induced current in our studies reversed at -85 mV, suggesting that the Y5R antagonist blocked activation of Kir or GIRK channels through the $G_{i/o}$ family of GPCRs [50,51]; these possibilities are currently being tested pharmacologically. Our results also showed that activation of GnRH neurons by blocking the Y5R required endogenous kisspeptin tone. It is interesting that antagonizing the Y5R resulted in a prolonged activation of the GnRH cell, similar to the effects of kisspeptin. If NPY's inhibitory actions are primarily through Kir or GIRK channels, then the actions of the Y5R antagonist would block these channels and perhaps reveal the excitatory actions of endogenous kisspeptin tone [27]. In speculating about the essential nature of kisspeptin's actions that are required for activation of the GnRH cell, it is possible that activation of TRPC channels may be a requisite component of GnRH cell firing, a component that is provided by endogenous kisspeptin tone [27].

Recent work from our group has identified CART as a novel stimulator of both kisspeptin and GnRH neurons [23]. CART neurons are expressed throughout the CNS and its mRNA and peptide have been identified in various hypothalamic regions [52,53]. Our work has shown that CART fibers are in close apposition to approximately 60% of GnRH neurons in the preoptic area [23]. In the present study, we found that CART induced depolarization in the majority of GnRH neurons, in keeping with the percentage of GnRH neurons contacted by CART fibers [23]. In addition, CART's effects were long-lasting, similar to the effects of kisspeptin [23,25]. Furthermore, our data suggest that CART's effects may be postsynaptic in nature on GnRH cells, although additional studies employing GABA and glutamate blockers, in addition to TTX, are necessary to provide definitive proof. The CART-induced increased action potential firing was consistently blocked by Peptide 347, suggesting an interaction between CART and kisspeptin. In considering possible mechanisms for this interaction, it is difficult to speculate because CART receptors have yet to be identified. Therefore, it is not possible to know whether CART's effects are on the same or different membrane channels as those for kisspeptin. However, a similar explanation as provided for the requirement of

endogenous kisspeptin tone for the excitatory effects of the Y5R could obtain here, that activation of TRPC channels by kisspeptin may be a requisite component of GnRH cell firing. Taken together, these findings portray the novelty and importance of endogenous kisspeptin tone for excitatory actions of neuropeptides on GnRH neurons. Studies in progress are exploring pharmacologically whether activation of TRPC channels is an essential component in GnRH activity.

Our findings provide an important analysis of the interaction between neural circuits controlling reproduction and feeding. Under states of negative energy balance, the orexigenic effects of NPY are increased, causing an increased inhibitory effect on GnRH neurons, resulting in a higher percentage of quiescent GnRH neurons [1,22]. In contrast, CART levels are decreased during states of negative energy balance [23,54,55]. In addition, it has been shown that lactation, fasting and caloric restriction lead to a reduction in kisspeptin in both ARH and AVPV [1,20,56,57,58]. Therefore, during conditions of negative energy balance, decreased kisspeptin tone, coupled with an increase in inhibitory NPY and a decrease in excitatory CART, could be major components in suppressed GnRH activity.

These studies also provided additional support for our earlier observations that there may be continued release of neuropeptides under conditions of TTX, as evidenced by the ability to demonstrate effects of the Y5R and kisspeptin antagonists (fig. 4, 6; [22]). The effects of TTX were evident by the absence of action potentials or spikes after treatment. Current dogma supports the idea that release of neuropeptides requires activated sodium channels, which are blocked under TTX. However, our data suggest that there may be continued presence of neuropeptides in the synapse and areas surrounding the GnRH neuron. Under our experimental conditions, the antagonists were effective even beyond 30 min of TTX exposure (data not shown), suggesting that there is a release component of neuropeptides that is not blocked by TTX. Since we observed these antagonist effects with both kisspeptin and NPY, antagonists to completely different receptors, this suggests that the phenomenon is not linked to a specific type of receptor or ligand. Support for the possibility of continued basal release of neuropeptides in the absence of activated sodium channels comes from studies of other neuropeptides in the hypothalamus, where continuous presence of oxytocin and vasopressin primes the dendrites in the supraoptic nucleus for release of vasopressin and oxytocin independent of electrical activity of the cell [59,60,61].

In conclusion, the current novel findings are derived from electrophysiological recordings of GnRH neurons and provide evidence supporting the widely held assumption that kisspeptin drives basal GnRH neuronal activity. Endogenous basal kisspeptin tone appears to be required for spontaneously activity in GnRH neurons, based on the results using a kisspeptin antagonist, Peptide 347, whose actions are consistent with blocking the Kiss1R. Furthermore, excitatory kisspeptin tone appears to be required for the excitatory effects of inhibition of Y5R or of CART on GnRH neurons. The requirement for kisspeptin tone may reflect the requirement for specific channel activation or inhibition that is necessary to activate GnRH neuronal firing. We also provide continuing evidence that there may be basal release of neuropeptides such as kisspeptin and NPY that is independent of the electrical activity of the cell. These studies present a possible model where endogenous kisspeptin tone could serve as the linchpin in GnRH activation or inhibition depending on the metabolic state of the animal.

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Disclosure Statement

The authors declare no competing financial interests.

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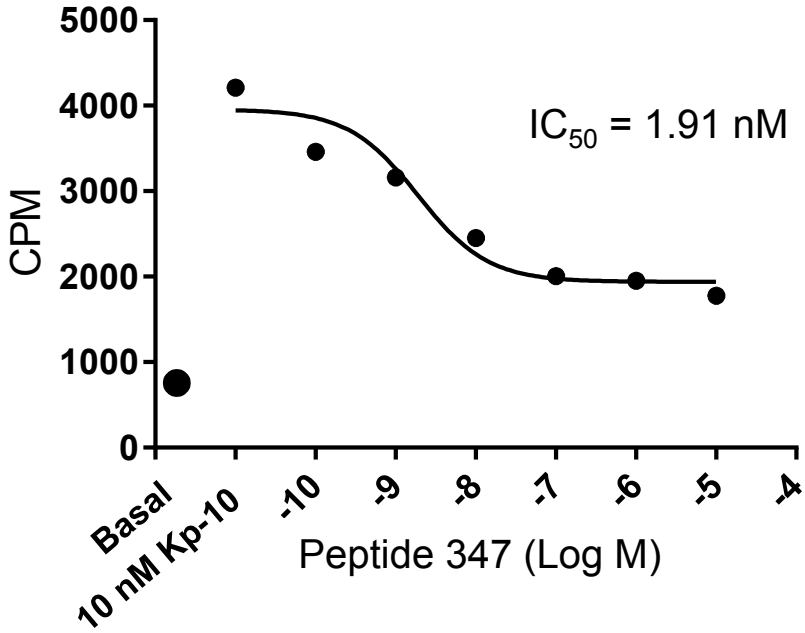
Supplemental Figure 1



Supplemental Figure 1. Peptide 347 inhibition of Kp-10 induced inositol phosphate production in CHO cells. Peptide 347 inhibition (IC_{50}) of Kp-10 (10 nM) stimulation of inositol phosphate in CHO cells transfected with the human Kiss1R. Radioactive inositol phosphate production was assessed using ^{3}H-myoinositol. Detailed information about the methods is presented below.

Methods for Supplemental Figure 1

Materials

Human Kp-10 and Peptide antagonist 347 were custom synthesized by EZBiolabs. The purity was >80% by HPLC analysis. The authenticity of peptides was confirmed by mass spectrometry. The source of all other reagents was Sigma-Aldrich.

Cell Culture

Chinese hamster ovary (CHO) cells stably-expressing the human GPR54 receptor (CHO/GPR54) were obtained from Prof. G. Vassart, Univ. Brussels. The cells were maintained in F12 Ham's nutrient mixture (Gibco) supplemented with 10% fetal calf serum, 2% glutamine and 1% penicillin (10,000 units/ml)/streptomycin (10,000 mg/ml) at 37ºC in a humidified 5% CO_2 atmosphere. COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco)) supplemented with 10% fetal calf serum, 2% glutamine and 1% penicillin (10,000 units/ml)/streptomycin (10,000 mg/ml) at 37ºC in a humidified 5% CO_2 atmosphere.

GPR54 Transfection

COS-7 cells were trypsinized and $1x10^6$ cells/ml place in DMEM and mixed with a 30 μls of the human GPR54-plasmid DNA. Then 300 μl were placed in each cuvette and then pulsed at 250 V and 960 uF using a Gene Pulser (Biorad) and incubated at room temperature for 15 minutes. Cells were then suspended in DMEM and plated into 12-well plates at $1x10^5$ cells/well.

Inositol Phosphate (IP) Stimulation Assay

Assays were performed as previously described (1,2). Prior to stimulation CHO/GPR54 cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS; without calcium or magnesium) then incubated overnight with ^{3}H-myoinositol, labeled HEPES-modified DMEM with 1% penicillin/streptomycin at 37º C. HEPES-modified DMEM supplemented with 1% penicillin/streptomycin and 1% lithium chloride (0.5 ml) was added to cells for 30 min at 37º C to block IP hydrolysis. Cells were then stimulated with 0.5 ml KP-10 (10nM) diluted at 1:100 in the above media for 1 h at 37º C, then with 10 mM formic acid at 4º C for 1 h to lyse cells. Lysates were transferred to plastic tubes containing 0.5 ml Dowex resin to bind the radioactive IP and the resin was then washed with 1 ml water. The resin was next washed with 60 mM ammonium formate/5 mM sodium tetraborate followed by 1 M ammonium formate/0.1 M formic acid to release the bound radiation. Then 800 µl of the radioactive solution were transferred to scintillation vials containing 2.5 ml scintillation fluid and radioactivity counted on a Beta counter for 60 sec. Experiments were repeated 3-5 times. IP production was plotted as mean values ± SEM and analyzed by using a two-way ANOVA followed by Bonferroni post hoc test ($p \geq 0.05$).

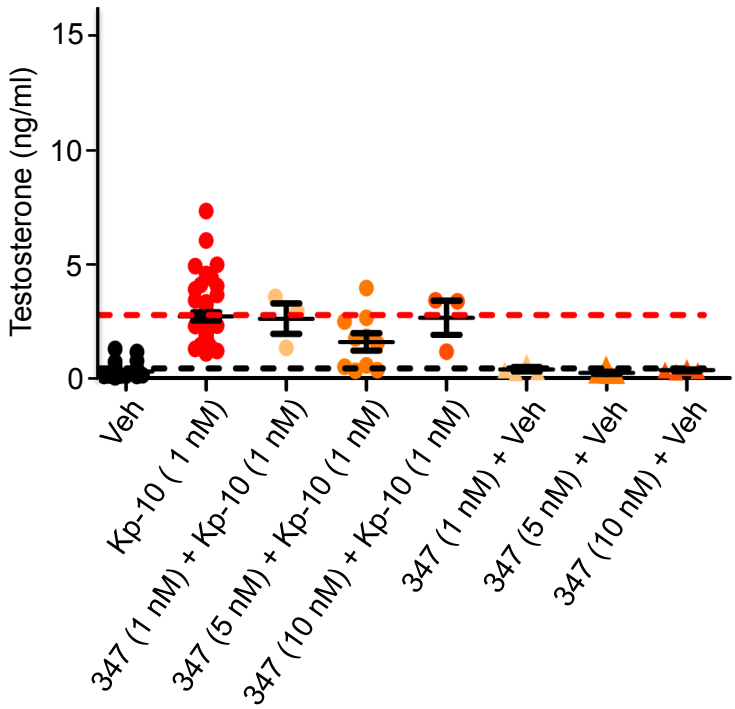
Inositol Phosphate (IP) Antagonism Assay

CHO/GPR54 cell monolayers were stimulated with 0.25 ml kisspeptin (10 nM) alone or in combination with 0.25 ml Peptide 347 (100 pM–1 µM), to investigate the inhibition of kisspeptin stimulation of IP production. Experiments were repeated 3 times. IP production was plotted as mean values ± SEM and analyzed using a two-way ANOVA followed by Bonferroni post hoc test ($p \geq 0.05$).

1. Coetsee M, Millar RP, Flanagan CA, Lu ZL: Identification of Tyr(290(6.58)) of the human gonadotropin-releasing hormone (GnRH) receptor as a contact residue for both GnRH I and GnRH II: Importance for high-affinity binding and receptor activation. Biochemistry 2008;47:10305-10313.

2. Lu ZL, Coetsee M, White CD, Millar RP: Structural determinants for ligand-receptor conformational selection in a peptide G-protein coupled receptor. J Biol Chem 2007;282:17921-17929.

Supplemental Figure 2



Supplemental Figure 2. Peptide 347 inhibits Kp-10 stimulation of testosterone in immature male mice. Peptide 347 was injected prior to and 30 min after administration of either vehicle or Kp-10 (10 nM). Peptide 347 significantly inhibited the testosterone response at all doses tested. Detailed information about the methods is presented below.

Methods for Supplemental Figure 2

Animals

Pre-pubertal male mice (30 days old) were purchased from Harlan UK and housed on a 14h light, 10h dark cycle (lights off at 1800h) in groups of 4-5 mice/cage. Rodent chow (Harlan Teklad 7912) and water were available *ad libitum*. All procedures were approved by the Animal Care and Use Committee of the University of Edinburgh.

Experimental Design

The effects of Peptide 347on the KP-10 stimulated testosterone secretion of pre-pubertal male animals were determined. Pre-pubertal male mice (30 day old) were given an i.p. injection (1 µl each) of either vehicle (10% DMSO) or Peptide 347 (1, 5 or 10 nmol). A second i.p. injection (1 µl each) of Peptide 347 was administered after 30 min of either vehicle or Kp-10 (1 nmol). Blood was collected via cardiac puncture 20 min after the second infusion and centrifuged for 10 min at 1000g. Plasma was collected and stored at -20C until assayed for testosterone. All infusions were performed between 1000h and 1400h. Groups contained n=8-12 animals.

Testosterone Measurements and Statistical Analyses

For all experiments, serum testosterone levels were measured by radioimmunoassay. Samples from each mouse were assayed in duplicate in a single assay, and the within-assay coefficients of variation were all below 10%. For each experiment, all samples were measured in the same assay. Values are presented as group means ± S.E., and are analyzed using a two-way ANOVA followed by Bonferroni post hoc test ($p \geq 0.05$).