RF9 Excitation of GnRH Neurons Is Dependent Upon Kiss1r in the Adult Male and Female Mouse

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The neuropeptide FF receptor antagonist 1-adamantanecarbonyl-Arg-Phe-NH2 trifluoroacetate salt (RF9) has been found to be a remarkably potent activator of gonadotropin secretion in mammals. However, the mechanism of RF9 action on the reproductive axis is unknown. Using acute brain slice electrophysiology in genetically modified mouse models, we have investigated the possibility that RF9 may activate GnRH neurons. In transgenic GnRH-GFP male and female mice, RF9 was found to exert potent, dose-dependent, stimulatory effects on the firing rate of approximately 70% of GnRH neurons. These effects occurred directly on GnRH neurons and were independent of fast amino acid transmission. To assess RF9's action as an neuropeptide FF receptor antagonist at the GnRH neuron, its ability to antagonize the inhibitory effects of RFamide-related peptide-3 on GnRH neuron firing was examined. RF9 exhibited variable ability to prevent the inhibitory effects of RFamide-related peptide-3 on GnRH neurons. Whole-cell recordings from GnRH neurons showed that RF9 generated an inward current in GnRH neurons reminiscent of that evoked by kisspeptin. We therefore examined RF9 actions in kisspeptin receptor knockout mice. RF9 was found to have no effects at all on GnRH neurons in GnRH-GFP;*Kiss1r*-null mice, although these cells exhibited normal intrinsic electrical properties and remained responsive to GABA and glutamate. This study reveals that RF9 directly activates GnRH neurons in the mouse and that this is dependent upon Kiss1r expression. **(*Endocrinology* 155: 4915–4924, 2014)**

Research over many years has indicated that a wide variety of classical neurotransmitters, neuropeptides, and other signaling molecules can modulate the activity of GnRH neurons to control gonadotropin secretion (1, 2). Even though some transmitters, such as kisspeptin, are widely acknowledged as being of critical importance to GnRH neuron behavior, the functional hierarchy of most inputs remain unknown. Learning the physiological role of each signaling molecule and their combinatorial actions on GnRH neurons is an important but challenging task.

It was recently reported that 1-adamantanecarbonyl-Arg-Phe-NH2 trifluoroacetate salt (RF9) given parenterally or i.c.v. evokes a large increase in LH and FSH secretion in mice, rats, and sheep, regardless of breeding season or gonadal status (3–5). These effects were similar in magnitude to those evoked by lower concentrations of kisspeptin, another member of the RFamide family (3–5). This was a very surprising result, because RF9 was developed as an antagonist of the neuropeptide FF receptors (Npffrs) (Npff1r; also known as Gpr147 and Npff2r; also known as Gpr74) that are activated by a range of neuropeptides, including RFamide-related peptide-3 (RFRP-3) (6). As such, this suggested that tonic RFRP-3 signaling somewhere within the GnRH neuronal network exerted a very potent inhibitory action on the excitability of GnRH neurons across a wide range of conditions. Acute brain slice studies had indicated that RFRP-3 exerted both inhibitory and stimulatory effects upon the firing rate of approximately 50% of GnRH neurons (7, 8). However, these effects of RFPR-3 on GnRH neuron excitability were modest compared with kisspeptin and did not easily explain how RF9 could exert kisspeptin-like stimulatory actions upon LH secretion.

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Abbreviations: aCSF, artificial cerebrospinal fluid; AP5, D-2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; GABAzine, 2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide; Kp-10, kisspeptin-10; Npffr, neuropeptide FF receptor; RF9, 1-adamantanecarbonyl-Arg-Phe-NH2 trifluoroacetate salt; RFRP, RFamide-related peptide; TTX, tetrodotoxin.

From both a clinical and agricultural perspective is important to understand the cellular mechanism of action of any compound that has profound effects upon gonadotropin secretion. We set out here to examine the actions of RF9 on the electrical activity of GnRH neurons in the intact male and female mouse. We first questioned whether RF9 was able to modulate GnRH neuron firing in acute brain slices prepared from adult GnRH-GFP mice. Second, we were interested to define, as far as possible given present reagents for RFRP pharmacology, the mechanism of RF9 action on GnRH neurons. This ultimately resulted in the demonstration that RF9 activates GnRH neurons directly but that it does so in a kisspeptin receptor (Kiss1r)-dependent manner.

Materials and Methods

Experimental animals

Adult male and female C57BL6 homozygous GnRH-GFP (9) and GnRH-GFP;*Kiss1r*-null mice (10) were housed under 12-hour light, 12-hour dark cycles (lights on at 7 AM) with ad libitum access to food and water. All experimentation was approved by the University of Otago Animal Welfare and Ethics Committee. The estrous cycle stage of female mice was determined by daily vaginal smear, with mice being killed for experiments between 10 and 11 AM.

Brain slice preparation and electrophysiology

Brain slices were prepared as reported previously (11). In brief, 250-μm-thick sagittal or coronal brain slices containing the preoptic area were cut with a vibratome (Leica VT1000S), from a brain block submerged in cooled (~4°C) cutting artificial cerebrospinal fluid (aCSF) equilibrated with 95% O_2 and 5% CO_2 and containing high (6mM) $MgCl_2$ and low (0.5mM) $CaCl_2$. Brain slices were then incubated for at least 1 hour in equilibrated (95% O_2 and 5% CO_2; 30°C) aCSF, containing 118mM NaCl, 3mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgCl_2$, 11mM D-glucose, 10mM HEPES, and 25mM $NaHCO_3$, before being transferred to a submerged recording chamber where they were perfused with aCSF at 2–3 mL/min maintained at 32 ± 1°C. Cell-attached recordings (current clamp with 0 holding current) of GnRH neurons were undertaken using a fixed-stage upright microscope (BX51WI; Olympus) under Nomarski differential interference contrast optics (a 40× water-immersion objective). Patch pipettes were pulled from glass capillaries (id, 1.17 mm; od, 1.5 mm) with a microelectrode puller (Sutter Instruments) and had 3- to 5-MOhm resistances when filled with the pipette solution composed of 145mM NaCl, 3mM KCl, 2.5mM $CaCl_2$, 10mM HEPES, and 1.2mM $MgCl_2$ (pH 7.35 adjusted by NaOH, and ~290mM mOsmol). For whole-cell recording, patch pipettes were filled with an internal solution including 128mM Kgluconate, 8mM KCl, 10mM HEPES, 0.4mM Na_2GTP, 4mM MgATP, 2mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 0.5mM $CaCl_2$, and 5mM phosphocreatine-Na_2 (pH 7.43 adjusted by KOH, ~290 mOsmol). Signals (voltage and current) were amplified with a Multiclamp 700B amplifier (CV7B; Molecular Devices) and sampled on-line with the use of a Digidata 1440A interface (Molecular Devices) connected to a personal computer. Signals were filtered (3 kHz for voltage clamp or 10 kHz for current clamp; Bessel filter of Multiclamp 700B) before being digitized at a rate of 1 kHz for axoscope recording or 10 kHz for pClampex recording. Acquisition and subsequent analysis of the acquired data were performed with the Clampex 10 suite of software (Molecular Devices) and Origin Pro 7.5 (OriginLab Corp).

Resting membrane potential was recorded in current clamp without applying any holding current and was not corrected for the liquid junction potentials of approximately 12 mV. The access and input resistances, membrane capacitance, and membrane time constant were determined by pClampex 10 membrane test while holding the cell at −60 mV. During experiments, the access resistance (Ra = 12 ± 1 MΩ; n = 36) was checked after each drug, and if Ra change during the drug was more than 20%, the datum was excluded for analysis.

To determine the reversal potential of RF9-induced postsynaptic inward current, a voltage step (from −130 to −30 mV, with 20 mV increments) protocol was given before, during, and after washout of RF9. The calculated K^+ equilibrium potential was −100 mV without correction for the mobility of potassium gluconate.

Drugs

Stock solutions of 20mM D-2-amino-5-phosphonovaleric acid (AP5), 10mM (RS)-baclofen, 1M glutamic acid, 5mM 2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide (GABAzine), 100mM muscimol, 10mM RF9, 2.5mM rat RFRP-3 (ANMEAGTMSHFPSLPQRFNH2; Sigma Ltd) and 1mM tetrodotoxin (TTX) (Alomone Labs) were prepared in double distilled H_2O. Stock solutions of 20mM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Sigma Ltd) and 2mM human kisspeptin-10 (Kp-10) (Calbiochem, EMD Biosciences) were prepared in DMSO (final concentration of DMSO was ~0.04%). Kynurenic acid (2mM; Sigma Ltd) was freshly prepared in aCSF on the experiment day. All stock solutions were stored at −20°C. All drugs were applied in the perfusion solution with the final concentration as indicated.

Analysis

The drug-induced changes in action potential frequency were determined as follows. Each action potential in the continuous voltage recordings acquired at 10 kHz was detected by Clampfit 10 and sorted into 10-s bins to generate frequency histograms for each cell. From this, the start and end of any response was determined. The end of the response was the time at which the firing rate of the cell returned to pretest levels. The 3-minute period immediately before administration of the drug was considered the "predrug interval." The entire period between the beginning and end of the response was considered the "drug interval," whereas the 3-minute period commencing immediately after the end of the response was considered the "postdrug interval." For cells in which the drug had no effect on firing, the start and end points of drug application were considered the start and end points of the "response." The percentage increase in firing was calculated as 100 × [(frequency of "drug" − frequency of "predrug")/frequency of "drug")]. If a silent GnRH neuron began to

fire, this was considered 100%. A GnRH neuron was considered to have responded to a compound if its percentage increase was more than 25% and a significant ($P < .05$) change was found comparing "drug interval" with "predrug" and "postdrug" intervals with Kruskal-Wallis ANOVA. Because individual GnRH neurons were tested with RF9 for varying periods of between 2 and 4 minutes, the duration of change in firing is also reported as the "duration of the response"/"RF9 application period."

The amplitudes of drug-induced currents were determined as follows: recordings of continuous currents acquired at 0.33 kHz were filtered by low-pass 10-Hz filter (Gaussian) in Clampfit 10 with the root of the mean square of the filtered continuous currents being 1.7 ± 0.2 pA (mean ± SD, n = 36). If the difference between the average of 1-minute drug-induced peak current and the average of predrug current (1 min) was more than 2× the SD of the predrug current, the difference was considered significant.

Statistical analysis was undertaken using Wilcoxon-signed ranks test for comparison between mean pretest and post-test group values. Kruskal-Wallis ANOVA, for the comparison of groups, and Fisher's exact test for comparing the frequency of responses between 2 groups, respectively. The statistical test is noted for each result.

Results

RF9 excites GnRH neurons in a dose-dependent manner

RF9 was tested at concentrations of 50nM, 200nM, and 1μM applied for 2–4 minutes to preoptic area GnRH neurons from adult male (n = 20, 11 animals) and diestrous female (n = 62, 24 animals) GnRH-GFP mice. The 1μM concentration employed here is calculated to approximate the minimum RF9 concentration needed to activate gonadotropin secretion in vivo (3). Cell-attached voltage recordings revealed that RF9 exerted a potent dose-dependent excitatory action on GnRH neurons (Figure 1). At 50nM, RF9 had no effects upon GnRH neuron firing (n = 8) (Figure 1, A, B, D, and E). At 200nM, RF9 excited 21 of 51 GnRH neurons (41%) (Figure 1) increasing firing frequency from 2.47 ± 0.53 to 3.95 ± 0.67 Hz ($P < .001$, Wilcoxon-signed ranks test) with the average duration of excitation lasting 7.7 ± 0.8 minutes. The ratio of RF9 response time to application time was 2.7 ± 0.3.

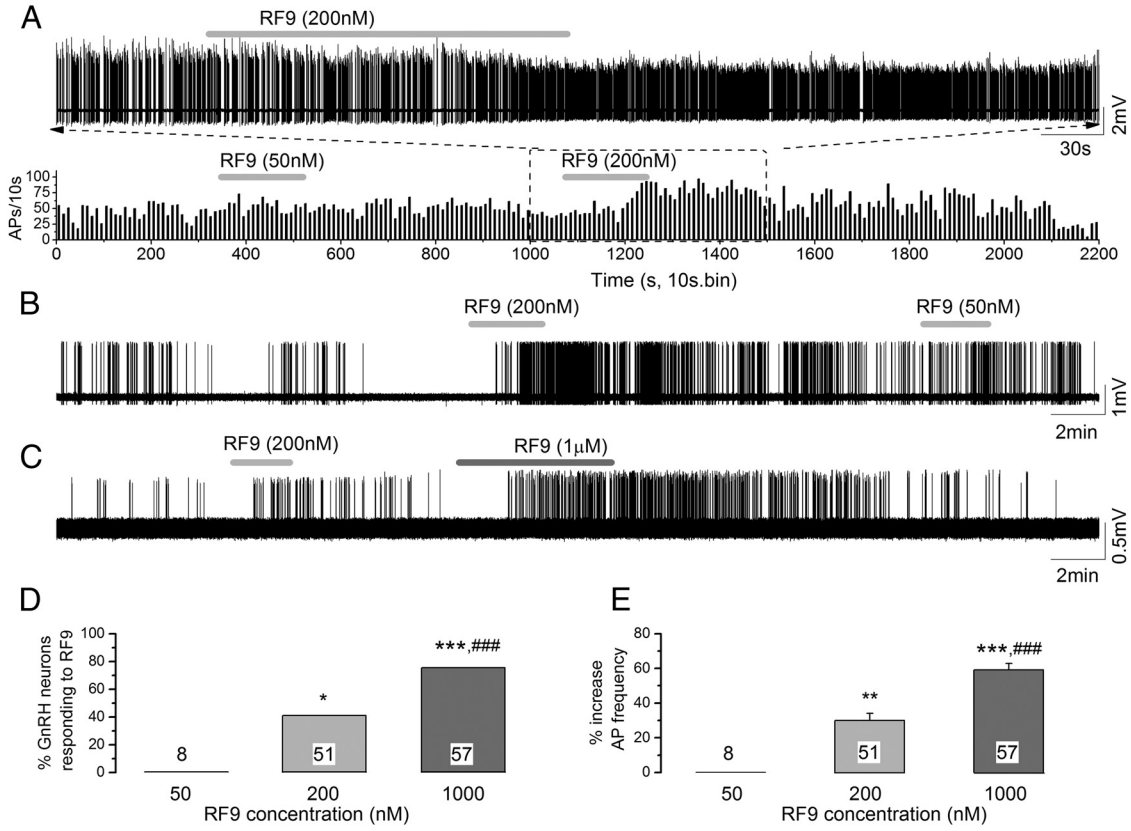

Figure 1. RF9 excites GnRH neurons in a dose-dependent manner. A, Frequency histogram (below) and portion of trace (above) from a cell-attached voltage recording of a GnRH neuron from an adult female diestrous GnRH-GFP mouse showing a dose-dependent activation of GnRH neuron firing by RF9. B and C, Two more cell-attached voltage recordings of adult female diestrous GnRH neurons also showing a dose-dependent activation by RF9. Note the difference in amplitude scale in B and C. D, Histogram showing the percentage of GnRH neurons responding to RF9 at the 3 different concentrations. E, Histogram showing the percentage increase in firing rate of GnRH neurons responding to RF9 at the 3 different concentrations. Numbers of GnRH neurons are given at the base of histograms. *, $P < .05$; **, $P < .01$; ***, $P < .001$, compared with RF9 50nM; ###, $P < .001$ compared with RF9 200nM. Fisher's exact test for D. One-way ANOVA with post hoc Bonferroni test for E.

RF9 at 1μM excited 43 of 57 (75%) GnRH neurons (Figure 1, C–E) increasing firing frequency from 1.59 ± 0.30 to 3.41 ± 0.50 Hz ($P < .001$, Wilcoxon-signed ranks test) with the average duration of excitation lasting 9.9 ± 0.7 minutes. The ratio of RF9 response time to application time was 2.8 ± 0.2. The effects of 1μM RF9 were not different ($P = .52$, Fisher's exact test) in GnRH neurons from males (overall, 14 of 17 [82%] excited) and females (overall, 29 of 40 [73%] excited). Six GnRH neurons tested sequentially with 200μM and 1μM RF9 exhibited dose-dependent increases in firing rate (Figure 1C), and as a group, a dose-dependent increase in firing was observed both in relation to the number of GnRH neurons activated by RF9 (Figure 1D) and their degree of activation (Figure 1E).

RF9 excites GnRH neurons independent of fast amino acid transmission

To examine whether RF9's excitatory actions on GnRH neurons (n = 6 male and n = 5 female) were dependent upon GABA and glutamate ionotropic receptor synaptic transmission, a cocktail of antagonists (2mM kynurenic acid, 20μM CNQX, and 5μM GABAzine) was applied in the bath before 1μM RF9 application. The antagonist cocktail did not block the response to RF9 (Figure 2). RF9 increased the frequency of GnRH neuron firing from 1.35 ± 0.40 to 2.34 ± 0.54 Hz (n = 11, $P < .01$, Wilcoxon-signed ranks test) in the absence of antagonist cocktail and from 1.42 ± 0.50 to 2.39 ± 0.44 Hz (n = 11, $P < .01$, Wilcoxon-signed ranks test) in the presence of antagonist cocktail. The duration of response to RF9 was 8.0 ± 1.6 minutes (control) and 8.6 ± 1.7 minutes (antagonist), with the ratio of RF9 response time to application time being 3.4 ± 0.7 (control) vs 3.7 ± 0.9 (antagonists; $P = .68$, Wilcoxon-signed ranks test). The RF9-induced increase in firing frequency was 56 ± 5% (control) vs 54 ± 5% (antagonist; $P = .756$ Wilcoxon-signed ranks test, n = 11).

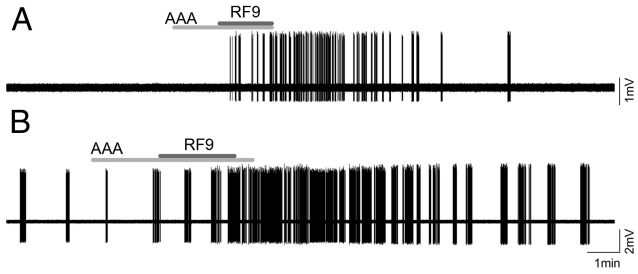

Figure 2. RF9 action on GnRH neurons is independent of ionotropic glutamate and GABA receptor transmission. Two cell-attached voltage recordings of GnRH neurons from female mice showing that RF9 (1μM) excited the neurons in the presence of a cocktail of amino acid receptor antagonists (AAA; 2mM kynurenic acid, 20μM CNQX, and 5μM GABAzine).

RF9 acts directly on GnRH neurons in manner similar to kisspeptin

To help elucidate the site and mechanism of RF9 action on GnRH neurons, whole-cell recordings were undertaken in 19 GnRH neurons. GnRH neurons exhibited a resting membrane potential of −58.8 ± 2.1 mV with input resistance of 882 ± 55 MOhm (Table 1). In the presence of 20μM AP5, 20μM CNQX, 5μM GABAzine, and 0.5μM TTX, 8 out of 19 GnRH neurons held at −60 mV responded to RF9 (1μM) with an inward current (peak current: 10.2 ± 1.9 pA, range from 4.6 to 20.7 pA; $P = .014$, Wilcoxon-signed ranks test) (Figure 3, A and B). The inward currents were accompanied by an increase in input resistance (Figure 3, C and D) and had a reversal potential of −95 mV (Figure 3C, inset) in 2 GnRH neurons. In the remaining 6 GnRH neurons the current evoked by RF9 approached 0 pA at −130 mV, although no absolute reversal potential was found at between −130 and −30 mV (Figure 3D, inset). These data show that RF9 acts directly on GnRH neurons and suggest that RF9 acts by blocking K^+ channels (reversal potential of −95 mV), and in most cells, this is accompanied by opening a nonselective cation channels (cells showing mixed current approaching reversal at −130 mV). This is similar to the action of kisspeptin on GnRH neurons (12, 13).

RF9 partly antagonizes actions of RFRP-3 on GnRH neurons

RF9 was developed as an antagonist of Npffrs (6) and GnRH neurons express Npff1r (5, 14) and respond to RFRP-3 (7, 8). To help assess the mechanism through which RF9 activates GnRH neurons, we examined the ability of 0.5μM and 1μM RF9 to antagonize the inhibitory actions of RFRP-3. Twenty GnRH neurons inhibited by 1μM RFRP-3 were identified and treated with 1μM RF9 2 minutes before or coincident with being retested with 1μM RFRP-3. As expected, most these cells were activated by RF9 (Figure 4, A–D), making it difficult to compare the degree of RFRP-3 inhibition before and after RF9 treatment, because the baseline firing rate was very different. Eight of 20 (40%) GnRH neurons continued to be inhibited by RFRP-3 in the presence of RF9 (Figure 4,

Table 1. Summary of GnRH Neuron Electrical Properties in *Kiss1r*-Null Mice

Electrical properties	Control (n = 19)	Kiss1r-null (n = 17)
Input resistance (MΩ, R_{in})	882 ± 55	921 ± 93
Membrane capacitance (pF, Cm)	21.6 ± 0.9	21.3 ± 1.0
Time constant (μs)	252 ± 13	265 ± 20
Rest membrane potential (mV)	−58.8 ± 2.1	−55.6 ± 2.1

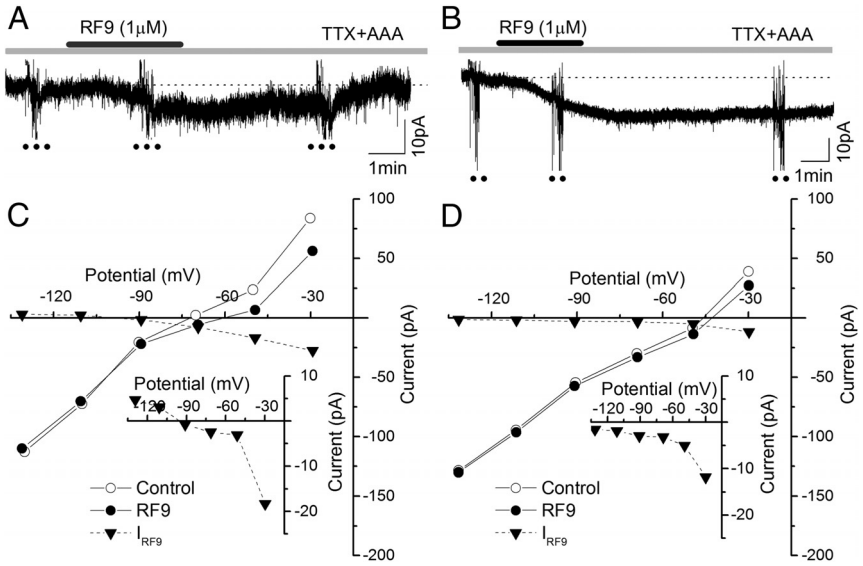

Figure 3. RF9 excites GnRH neurons by closing K+ and/or opening nonselective cation channels. A and B, Whole-cell current recordings from 2 GnRH neurons showing that RF9 (1μM) induces an inward current (holding potential −60 mV) in the presence of an amino acid receptor antagonist cocktail (TTX+AAA; 20μM AP5, 20μM CNQX, 5μM GABAzine, and 0.5μM TTX). Black dots indicate when series of voltage step currents were applied (truncated). C and D, Current-voltage plots before (control, open circles) and during RF9 application (RF9, filled circles) taken from the cells shown in A and B above, respectively. The "RF9 current" (I_{RF9}, filled triangles) is obtained by the subtraction of control from RF9 currents at each holding potential. For clarity, the RF9 currents in C and D are replotted as insets with an expanded current axis.

D–F). The remaining 12 GnRH neurons exhibited very reduced or no (Figure 4, A and B) response to RFRP-3 in the presence of RF9. The level of basal firing evoked by RF9 did not correlate with RFRP-3's ability to retain its inhibitory response; GnRH neurons highly activated by RF9 could respond (Figure 4, D and F) or not (Figure 4, A and C) to RFRP-3. Studies examining the effects of 500nM RF9 on 1μM RFRP-3 found the same effects. Three of 6 (50%) RFRP-3-sensitive GnRH neurons exhibited no response to RFRP-3 in the presence of 500nM RF9 (Figure 4C). Combined data from these 3 neurons (from 3 mice) showed that RFRP-3 alone decreased GnRH neuron firing from 0.42 ± 0.12 to 0.16 ± 0.05 Hz ($P < .05$, Kruskal-Wallis ANOVA test), whereas in the presence of 500nM RF9, firing was greatly elevated from 0.54 ± 0.17 to 1.74 ± 0.16 Hz (n = 3, $P = .05$, Kruskal-Wallis ANOVA test).

RF9 fails to activate GnRH neurons in Gpr54KO mice

Experiments above indicated that RF9 was a potent direct activator of GnRH neurons and that it did so in a manner reminiscent of kisspeptin. As such, we tested whether RF9 would have any effects on GnRH neurons in GnRH-GFP;*Kiss1r*-null mice (10). RF9 (1μM) was applied for 2–4 minutes to 17 GnRH neurons (n = 5 male, n = 12 female) in GnRH-GFP;*Kiss1r*-null mice. Cell-attached voltage recordings revealed that none of 17 GnRH neurons responded to RF9 or kisspeptin, although they still responded to 12mM KCl (Figure 5, A and B1–B3). In a second experiment, whole-cell current recordings in the presence of 20μM AP5, 20μM CNQX, 5μM GABAzine, and 0.5μM TTX were made from another group of 17 GnRH neurons (8 male, 9 female) from GnRH-GFP;*Kiss1r*-null mice. None of these cells showed any response to 1μM RF9 (−0.9 ± 0.5 pA; $P = .13$, Wilcoxon-signed ranks test) (Figure 5, C–E).

GnRH neurons in Kiss1r-null mice continue to respond to amino acid transmitters

The above experiment revealed that Kiss1r was essential for RF9 to activate GnRH neurons. This may result from RF9 acting on Kiss1r to activate GnRH neurons or RF9 acting on GnRH neurons that are generally dysfunctional in the absence of kisspeptin inputs (1). To test the electrical integrity of GnRH neurons in the absence of kisspeptin inputs, we evaluated their intrinsic electrical properties and responses to the amino acid transmitters in *Kiss1r*-null mice.

Whole-cell recordings were made from 19 GnRH neurons (5 male, 12 female) in GnRH-GFP mice (control) and 17 GnRH neurons (8 male, 9 female) in GnRH-GFP; Kiss1r-null (Kiss1r-null) mice. Input resistance (882 ± 55 [control] vs 921 ± 93 MΩ [Kiss1r-null]), membrane capacitance (21.6 ± 0.9 [control] vs 21.3 ± 1.0 pF [Kiss1r-null]), membrane time constant (252 ± 13 [control] vs 265 ± 20 μs [Kiss1r-null]), and resting membrane potential (−58.8 ± 2.1 [control] vs −55.6 ± 2.1 mV [Kiss1r-null]) were not statistically different ($P > .05$, Kruskal-Wallis ANOVA) (Table 1). GnRH neurons were tested with bath-applied baclofen (10μM), glutamate (200μM), and muscimol (25μM). Six out of 9 control (67%) and 9 out of 13 Kiss1r-null (69%) GnRH neurons responded to glutamate with an inward current held at −60 mV (39.7 ± 20 [control] vs 21.3 ± 3.4 pA [Kiss1r-null]) (Figure 6, C and D). Nine out of 9 control (100%) and 5 out of 7 Kiss1r-null (71%) GnRH neurons responded to muscimol with an inward current held at −60 mV (21.1 ± 2.0 [control] vs 38.8 ± 14.6 pA [Kiss1r-null]) (Figure 6, E and F). Nine out of 9 control (100%) and 8 out of 11 Kiss1r-null

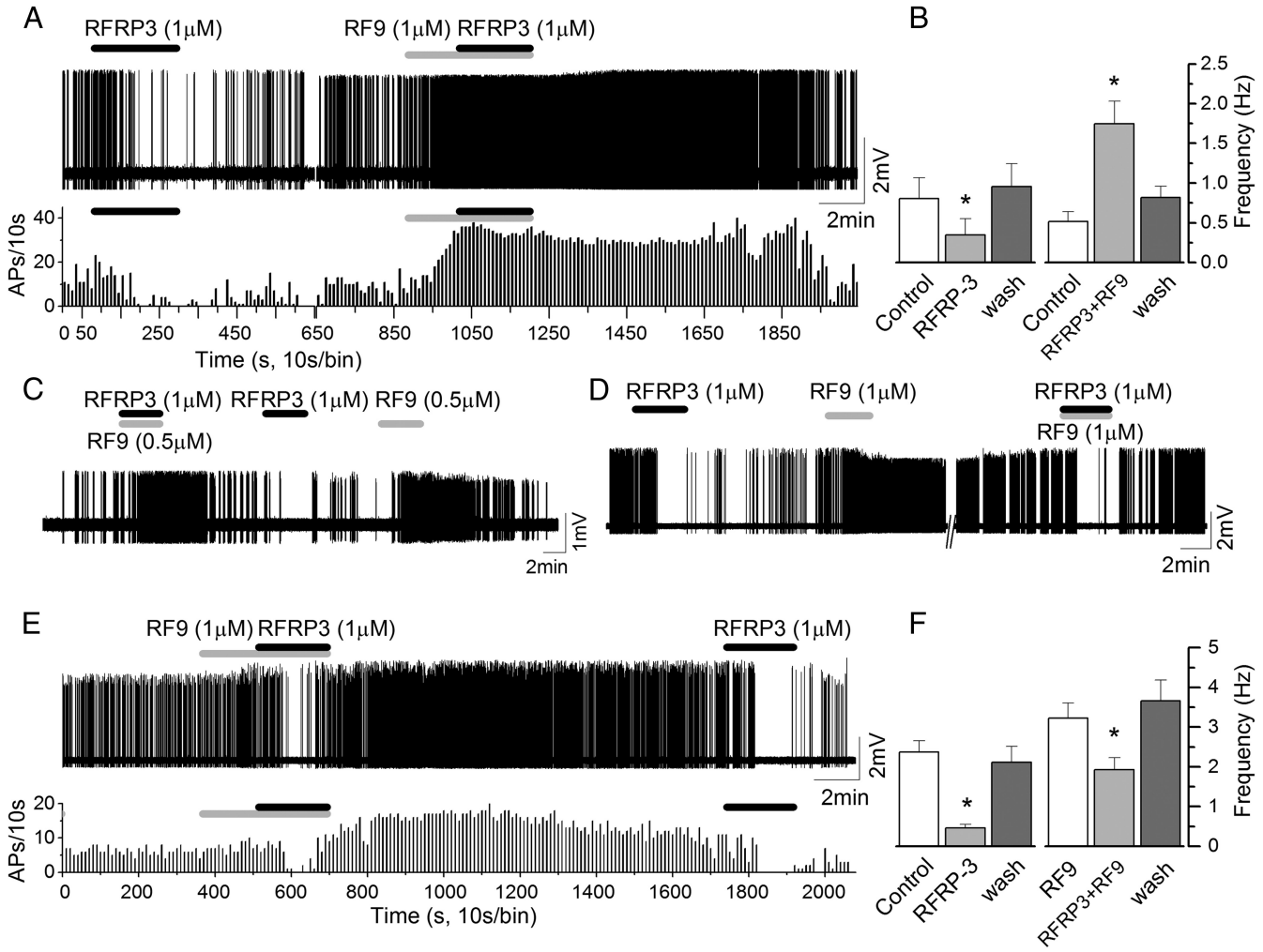

Figure 4. RFRP-3 has variable ability to inhibit GnRH neuron firing in the presence of RF9. A and C, Cell-attached voltage recordings (with frequency histogram below) of 2 GnRH neurons from 2 male mice that were inhibited by RFRP-3 (RFRP3) and activated by RF9 but then failed to show RFRP-3 inhibition in the presence of RF9. B, Histograms summarize 12 GnRH neurons in which inhibitory responses to RFRP-3 were absent in the presence of 1μM RF9. D and E, Two examples of GnRH neurons from diestrous mice in which RF9 activates GnRH neurons but in which RFRP-3 remains able to suppress firing rate. Firing rate histogram is shown below E. F, Histograms summarize 8 GnRH neurons in which the responses to RFRP-3 are not blocked by RF9, as shown in D and E. *, $P < .05$; compared with control and wash (Kruskal-Wallis ANOVA test).

(73%) GnRH neurons responded to baclofen with an outward current held at −60 mV (16.4 ± 2.0 [control] vs 11.6 ± 1.8 pA [Kiss1r-null]) (Figure 6, A and B). Neither the percentage of responsiveness to baclofen, glutamate, and muscimol (Figure 6G) nor the peak amplitude of current (Figure 6H) was statistically different ($P > .05$, Kruskal-Wallis ANOVA).

Discussion

We report here that 1μM RF9, a concentration that activates gonadotropin secretion in vivo, exerts direct stimulatory actions upon the electrical excitability of approximately 70% of GnRH neurons in the adult male and female mouse brain. Only limited evidence was found for 1μM RF9 being an antagonist of Npff receptors in GnRH neurons. In contrast, whole-cell recordings of GnRH neurons indicated that RF9 activated currents similar to those coupled to Kiss1r. In examining the possibility that RF9 activated Kiss1r to stimulate GnRH neurons, we found that RF9's actions on GnRH neurons disappeared completely in *Kiss1r* null mice. Together, these data indicate a critical role for Kiss1r in the ability of RF9 to activate GnRH neurons to stimulate gonadotropin secretion.

Studies in vivo have found robust effects of RF9 on gonadotropin secretion (3–5). The study by Pineda et al (3) demonstrated a dose-dependent increase in LH secretion with the minimal effective i.c.v. dose in male rats being 1 nmol RF9. Given a rat brain CSF volume of approximately 300 μL (15), this would have resulted in RF9 concentrations of approximately 3μM within the brain. We report here dose-dependent actions of RF9 in a similar range

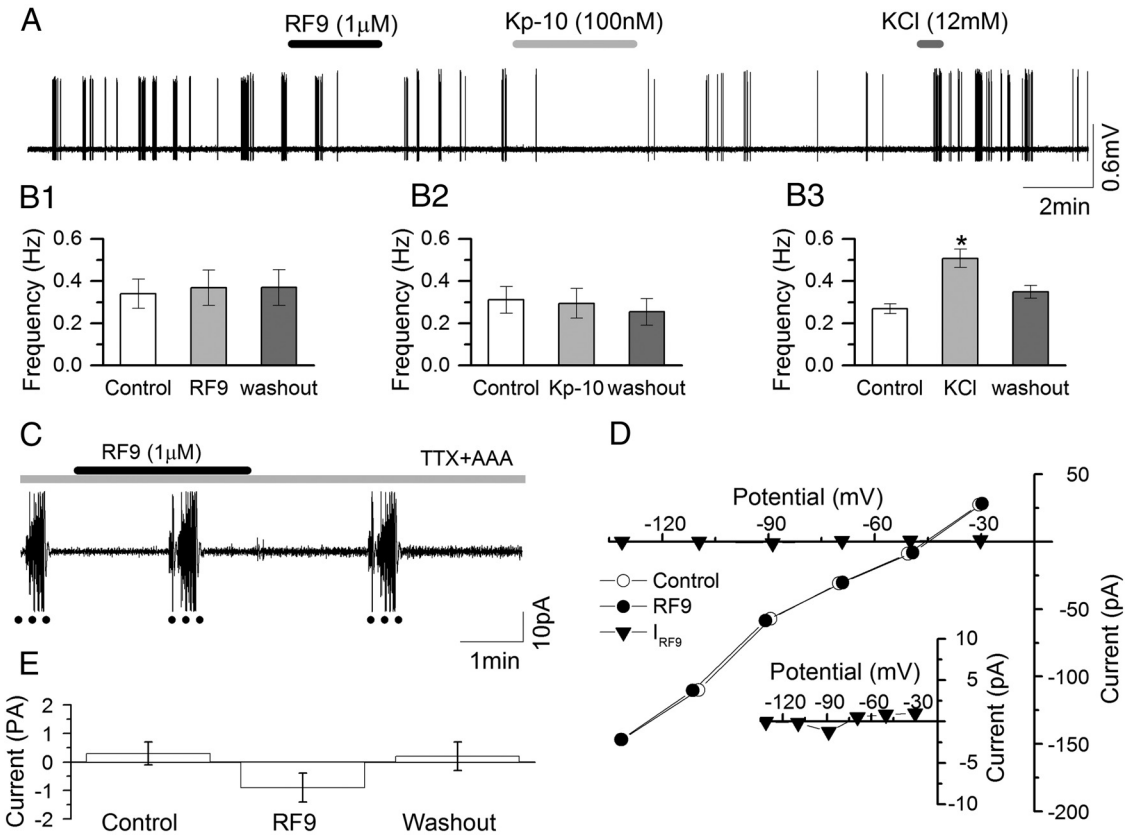

Figure 5. RF9 fails to activate GnRH neurons in GnRH-GFP;Kiss1r-null mice. A, Cell-attached voltage recording of a GnRH neuron from a female GnRH-GFP;Kiss1r-null mouse showing that both RF9 and kisspeptin (Kp-10) had no effect on firing despite 12mM potassium being effective. B1–B3, Histograms summarize 17 GnRH neuron responses to RF9, Kp-10, and KCl, respectively. C, A whole-cell current recording of a GnRH neuron from a female GnRH-GFP;Kiss1r-null mouse in the presence of an antagonist cocktail (TTX+AAA; 20μM AP5, 20μM CNQX, 5μM GABAzine, and 0.5μM TTX) showing that RF9 had no effect (holding potential was −60 mV). Black dots indicate when series of voltage step currents were applied (truncated). D, Current-voltage plots before (control, open circles) and during RF9 application (RF9, filled circles) taken from the cell shown in C. RF9-induced current (I_{RF9}, filled triangles) was obtained by the subtraction of currents during control at each holding potential from the currents during RF9 at corresponding holding potentials. For clarity, the RF9 currents (I_{RF9}, filled triangles) in D are also replotted as an inset with an expanded current axis. E, Histogram summarizes RF9 actions on current in17 GnRH neurons in GnRH-GFP;Kiss1r-null mice. *, $P < .05$; compared with control and wash. One-way ANOVA with post hoc Fisher's least significant difference (LSD) test.

within the acute brain slice; 50nM had no effect, 200nM activated 41% of GnRH neurons, whereas 1μM stimulated 73% of cells. As shown in the amino acid antagonist cocktail study and whole-cell recordings, that electrically isolate the recorded GnRH neuron, effects of RF9 are direct on GnRH neurons. This is consistent with in vivo studies reporting that the stimulatory actions of RF9 on gonadotropin secretion are dependent upon GnRH (3, 5). Thus, the ability of RF9 to modulate gonadotropin secretion is likely to occur, at least in part, through direct actions on GnRH neurons.

The mechanism of RF9 activation of GnRH neurons appears to be multifactorial. As an antagonist of Npff receptors (6), RF9 was initially thought to act by suppressing on-going inhibitory actions of RFRP-3 on GnRH neurons mediated by Npff1r (5, 7, 8). Although the present study does not exclude completely this possibility, it seems unlikely to be the primary mechanism. First, we demonstrate that up to 70% of GnRH neurons are excited by RF9, and yet only 20%–30% of GnRH neurons are reported to express Npff1r mRNA (5, 14). Although electrophysiological studies have reported that 40%–50% of GnRH neurons are inhibited by RFRP-3 (7, 8), it is not known whether these actions are direct. This suggests that there is a substantial population of GnRH neurons that is not modulated directly by RFRP-3 but nevertheless responds directly to RF9. As such, heterodimerization of Npffr and Kiss1r, or interactions between their intracellular signaling cascades, cannot account for RF9 actions in most GnRH neurons. Second, our electrophysiological studies indicate that 0.5μM–1μM RF9 does not appear to be a strong antagonist of RFRP-3 actions on GnRH neurons. RF9 either failed to antagonize RFRP-3 inhibitory actions or activated the GnRH neuron so that RFRP-3 effects were difficult to discern. Although this leaves open the possibility that RF9 may indeed be acting as an antagonist at some GnRH neurons, the potent excitatory actions of RF9 make this difficult to demonstrate exper-

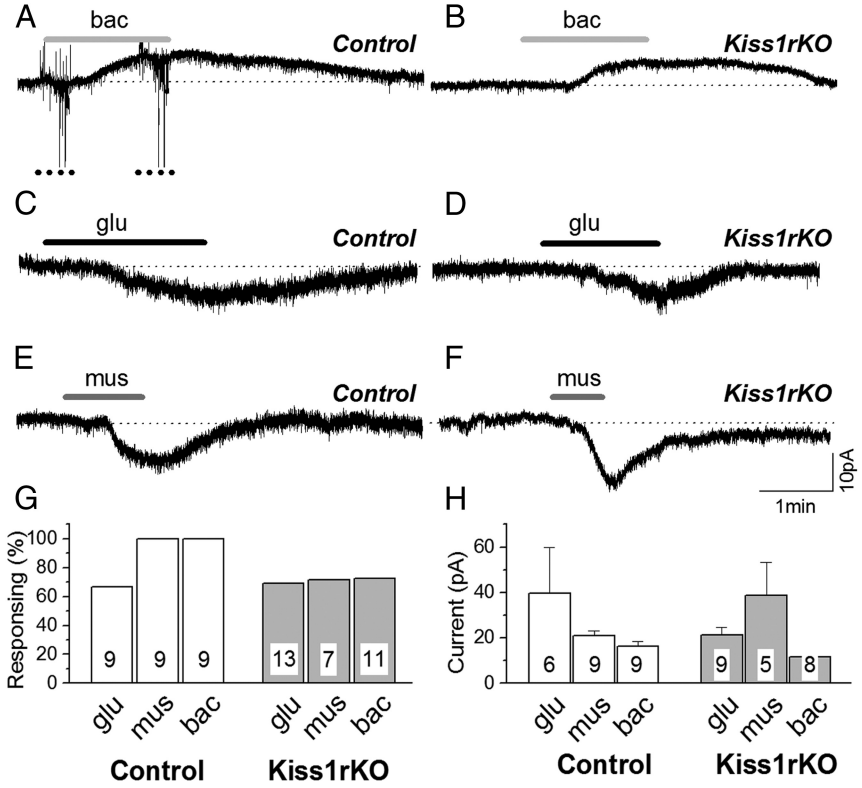

Figure 6. Responses of GnRH neurons to amino acids in GnRH-GFP;Kiss1r-null (Kiss1rKO) mice. A, C, and E, Whole-cell current recordings of GnRH neurons in control GnRH-GFP mice showing current responses to baclofen (bac; 10μM), glutamate (glu; 200μM), and muscimol (mus; 25μM). Voltage step-induced currents (truncated) are indicated by black dots. B, D, and F, Whole-cell current recordings of GnRH neurons from Kiss1rKO mice showing current responses to bac, glu, and mus. G, Histograms showing GnRH neuron response rates to bac, glu, and mus in GnRH-GFP (control) and Kiss1rKO mice. H, Histograms showing the peak currents induced by bac, glu, and mus in GnRH-GFP (control) and Kiss1rKO mice. Numbers of GnRH neurons are noted at the base of each histogram.

imentally. Additionally, we note that given reported Ki values (6, 20), 10- to 100-fold higher concentrations of RF9 over RFRP-3 may be required to fully assess antagonistic effects of RF9 at Npff receptors. Third, RFRP-3 inhibits GnRH neurons through opening potassium channels (8) so application of RF9 to block on-going RFRP-3 transmission in the slice should result in the closure of potassium channels. Although 2 GnRH neurons did show evidence of isolated closure of potassium channels, most responsive GnRH neurons exhibited I–V curves incompatible with RF9 acting only on potassium channels. Finally, we note that all of the RFRP-3 inputs to GnRH neurons will be severed in the coronal brain slice, making it very unlikely that any on-going RFRP-3 transmission exists for RF9 to antagonize in this preparation. Overall, these observations make it difficult to envisage how RF9 could activate GnRH neurons and, thereby LH secretion, by suppressing tonic RFRP-3 signaling.

We present evidence indicating that RF9 activates GnRH neurons primarily by acting through Kiss1r. First, we note that RF9 can activate up to 70% of GnRH neurons, similar to the numbers of cells (~85%) activated by kisspeptin (16). Second, when undertaking whole-cell experiments, we find evidence for RF9 acting through multiple conductances to drive an inward depolarizing current in GnRH neurons. This situation is similar to what is found for kisspeptin (12, 13, 17), with the closure of a potassium channel and involvement of nonselective cation channels (16). Third, and most importantly, we find that RF9 has no effects on GnRH neurons in mice lacking Kiss1r. This result is consistent with a previous in vivo study, in which i.c.v. RF9 was found to be unable to alter LH secretion in gonadectomized, testosterone treated Kiss1r null mice (18) and evoked less than 10% of the normal LH response in intact male *Kiss1r* null mice. It is also extremely unlikely that the absence of RF9 action in Kiss1r mice results from gonadal steroid modulation of Npff1r expression, because mRNA levels for the receptor in GnRH neurons are not reported to change after gonadectomy (14). Together, these studies show that kisspeptin signaling is essential for the stimulatory actions of RF9 on GnRH neurons and gonadotropin secretion.

The lack of RF9 response in *Kiss1r*-null mice may result from 1) developmental defects in the GnRH neuronal network of *Kiss1r*-null mice, that never exhibit full sexual maturity; 2) the requirement for kisspeptin signaling at the GnRH neurons to enable other neurotransmitters to be fully effective (parallel modulation); or 3) the possibility that RF9 acts directly on Kiss1r to activate GnRH neurons. There is evidence that GnRH neurons fire more slowly in the absence of kisspeptin inputs (10); and several neurotransmitters given i.c.v. are markedly less effective at activating LH secretion in *Kiss1r* null mice (18). To address the potential of "developmental defects" and "parallel modulation" to underlie the absent RF9 responses of GnRH neurons in *Kiss1r*-null mice, we examined the electrical properties of GnRH neurons and their responses to ionotropic and/or metabotropic GABA and glutamate transmission in *Kiss1r*-null mice. If these parameters, as general markers of GnRH neuron electrophysiological

"health," were abnormal in *Kiss1r*-null mice, then we could potentially explain the loss of RF9 actions. However, we found that the intrinsic membrane properties of GnRH neurons and their response to the amino acid neurotransmitters were normal in *Kiss1r*-null mice. This suggests that the lack of RF9 actions is not secondary to developmental or parallel defects in GnRH neuron excitability as adults. Of note, previous i.c.v. studies have variously shown impaired (18) or normal (19) amino acid regulation of LH in *Kiss1r*-null mice. Although the present study does not provide an explanation for the reduced firing rate of GnRH neuron in these mutants (10), it does indicate that GnRH neurons are not fundamentally impaired in their ability to respond to neurotransmitters. This supports the notion that the inability of RF9 to activate GnRH neurons in *Kiss1r*-null mice results from the absence of Kiss1r itself.

Brought together, the observations presented here suggest that RF9 may activate kisspeptin receptors on GnRH neurons to increase their excitability. The original studies undertaken by Simonin et al (6) reported that RF9 up to 10μM did not bind Kiss1r. However, recent studies have found different binding and kinetic properties of RF9 at Npff receptors (20), and there is increasing recognition that substantial crossover exists between the different RF-amide ligands and receptors (21). Nevertheless, it remains unclear how RF9 may interact with Kiss1r. It is notable that the duration of activation of GnRH neurons by 1μM RF9 is approximately 10 minutes, whereas 100nM kisspeptin generates responses that last for approximately 20 minutes (12). This may result from partial agonistic activity or allosteric modulation of Kiss1r by RF9. In any case, it is important to note that the actions of RF9 on LH secretion are likely to represent a pharmacological activation of kisspeptin receptors rather than delineating any physiological role for Npff receptors.

In conclusion, we report here that 1μM RF9 is a potent, direct activator of murine GnRH neurons and suggest that this likely underlies the ability of peripheral and central RF9 administration to strongly elevate gonadotropin secretion in mice (3). Studies will need to be undertaken in other mammals to establish the generality of this concept. Although it still remains possible that RF9 may activate GnRH secretion in vivo by antagonizing inhibitory RFRP-3 signaling at a small number of GnRH neurons, the principal mechanism of action would appear to involve a direct activation of GnRH neurons that is dependent upon Kiss1r.

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