Kisspeptin Regulation of Arcuate Neuron Excitability in Kisspeptin Receptor Knockout Mice

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The G protein-coupled receptor 54 (GPR54) is critical for kisspeptin to activate GnRH neurons to modulate fertility. However, the often mismatching distribution of kisspeptin and GPR54 in the brain suggests that kisspeptin may also act on other receptors. The arcuate nucleus (ARN) is one brain region with a very high density of kisspeptin fibers but only limited evidence for the expression of GPR54. Using acute brain slice electrophysiology in combination with *Gpr54* knockout (GPR54KO) mouse models, we examined whether actions of kisspeptin in the ARN were dependent upon GPR54. Cell-attached recordings from unidentified ARN neurons in wild-type mice revealed that approximately one third of neurons were either excited or inhibited by kisspeptin in a dose-dependent manner. The responses of ARN neurons to kisspeptin were exactly the same in GPR54KO mice despite effects of kisspeptin on GnRH neurons being abolished. To evaluate whether kisspeptin may be acting through neuropeptide FF receptors, the effects of an agonist RFamide-related peptide 3 (RFRP-3) and antagonists RF9 and BIBP-3226 were evaluated. Both the excitatory and inhibitory effects of kisspeptin were mimicked by the agonist RFRP-3. RF9 itself activated ARN neurons and suppressed only the inhibitory actions of kisspeptin. BIBP-3226 suppressed kisspeptin actions in 50% of neurons. Whole-cell recordings in GPR54KO mice demonstrated that both kisspeptin and RFRP-3 acted directly on the same ARN neurons and activated the same ion channels. Together, these studies demonstrate that kisspeptin can act partly through neuropeptide FF receptors to modulate neuronal activity independent of GPR54 in the mouse brain. **(*Endocrinology* 156: 1815–1827, 2015)**

Kisspeptin, a member of the RFamide family of peptides and the endogenous ligand for GPR54, is now well established as an important activator of GnRH neurons in the control of mammalian fertility (1–3). Many studies have documented the potent stimulatory influence of kisspeptin on the excitability of GnRH neurons both in vitro (4–8) and more recently in vivo (9). However, GPR54 is found in multiple brain regions as well as in peripheral tissues (10–12), indicating that kisspeptin-GPR54 signaling is not restricted to GnRH neurons. Indeed, within the brain, electrophysiological studies have reported actions of kisspeptin on neurons located in the hippocampus (13), preoptic area (7), and also in the arcuate nucleus (ARN) of the hypothalamus (14). The nature and roles of kisspeptin actions on non-GnRH neurons and in other tissues remain poorly understood at present.

Recent electrophysiological studies have demonstrated direct actions of kisspeptin on the electrical activity of proopiomelanocortin (POMC) neurons in the ARN (14). Experiments in vivo have also suggested that kisspeptin may have a role in the neural control of metabolism (15). Curiously, the ARN is a brain area with a very high density of kisspeptin fibers but only limited evidence for the expression of *Gpr54*. Although *Gpr54* mRNA has been reported in the mouse ARN with RT-PCR (14), a study examining the locations of *Gpr54* transcription in a

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Abbreviations: aCSF, artificial cerebrospinal fluid; AP, action potential; ARN, arcuate nucleus; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DMSO, dimethyl sulfoxide; GABAzine, 2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl) pyridazinium bromide; GFP, Green fluoresecent protein; GPR54, G protein-coupled receptor 54; GPR54KO, Gpr54-null; Kp-10, kisspeptin-10; NPFF, neuropeptide FF; NPY, neuropeptide Y; POMC, proopiomelanocortin; PrRP, prolactin-releasing peptide; RF9, 1-adamantanecarbonyl-Arg-Phe-NH2 trifluoroacetatesalt; RFRP-3, RFamide-related peptide 3.

Gpr54-LacZ knock-in mouse line failed to detect evidence for expression of *Gpr54* in the mouse ARN (10).

In the present study we have reexamined the electrophysiological actions of kisspeptin in the ARN and questioned specifically whether they are dependent upon GPR54 by comparing actions of kisspeptin in wild-type and *Gpr54*-null (GPR54KO) mice. We report here that kisspeptin acts independently of GPR54 to modulate electrical activity in the ARN and these effects may occur through the neuropeptide FF (NPFF) receptors.

Materials and Methods

Experimental animals

Adult C57BL/6J homozygous GnRH-GFP mice (16) and heterozygous GPR54 knockout mice (17) were housed under 12-hour light, 12-hour dark cycles (lights on at 7 AM) with ad libitum access to food and water. The mouse lines were crossed to generate $Gpr54^{-/-}$ (GPR54KO) and GnRH-GFP$^{+/-}$;$Gpr54^{-/-}$ (GnRH-GFP;GPR54KO) mice in addition to GnRH-GFP; GnRH-Cre;$Gpr54^{loxp/loxp}$ (GnRH neuron-specific GPR54KO) mice (3). 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 male and diestrous female mice killed between 10 and 11 AM.

Brain slice preparation and electrophysiology

Mice were decapitated, the brain quickly removed, and a block containing the hypothalamus submerged in cooled (~4°C) artificial cerebrospinal fluid (aCSF) equilibrated with 95% O_2, 5% CO_2 and containing high (6mM) $MgCl_2$ and low (0.5mM) $CaCl_2$. Coronal brain slices (250 μm thick) of the rostral preoptic area (for GnRH neuron recordings) and/or throughout the rostrocaudal extent of the ARN were cut with a vibratome (Leica VT1000S). Brain slices were incubated for at least 1 hour in equilibrated (95% O_2, 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 22 ± 1°C. Cell-attached recordings were undertaken using a fixed-stage upright microscope (BX51WI; Olympus) under Nomarski differential interference contrast optics (a ×40 water-immersion objective). Recordings from GFP-expressing GnRH neurons were undertaken as reported previously (18). Patch pipettes were pulled from glass capillaries (inner diameter, 1.17 mm; outside diameter, 1.5 mm) with a microelectrode puller (Sutter Instruments) and had 2–3 MΩ resistances when filled with the pipette solution composed of 145mM NaCl, 3mM KCl, 2.5mM $CaCl_2$, 10mM HEPES, 1.2mM $MgCl_2$ (pH 7.32 adjusted by NaOH, ~290 mOsmol) for cell-attached recordings. If the seal resistance of cell-attached recordings was less than 100 MΩ (16 ± 1 MΩ; range, 5–100 MΩ; n = 203), voltage recordings in current clamp mode with 0 holding current were used to minimize the electrode-induced impact on cell activity. If the seal resistance was more than 100 MΩ (2100 ± 250 MΩ; range, 450–4000 MΩ; n = 30), current recordings in voltage clamp with 0 holding voltage were used to generate lower noise recordings. For whole-cell recordings, patch pipettes (3–5 MΩ) were filled with a pipette solution composed of 125mM Kgluconate, 0.5mM $CaCl_2$, 10mM HEPES, 10mM EGTA, 4mM MgATP, 0.4mM Na_2GTP, and 5mM phosphocreatine-Na_2, (pH 7.3 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 or 10 kHz; Bessel filter of Multiclamp 700B) before being digitized at a rate of 1 kHz (Axoscope recording) or 10 kHz (pClampex recording). Data acquisition and analysis were performed with the Clampex 10 suite of software (Molecular Devices) and Origin Pro 7.5 (OriginLab Corp).

Drugs

Stock solutions of BIBP-3226 (BS11 0QL; Tocris Bioscience), RF9 (1-adamantanecarbonyl-Arg-Phe-NH2 trifluoroacetate salt), and GABAzine (2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide) (Sigma Ltd) were prepared in double distilled H_2O. Stock solutions of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) (Sigma Ltd) were prepared in DMSO (final concentration of DMSO in bath was ~0.04%). Stock solutions of rat RFamide-related peptide 3 (RFRP-3) (19) (ANMEAGTMSHFPSLPQRFNH2; synthesized by Sigma-Genosys, Sigma-Aldrich Corp, gift of Greg Anderson) and tetrodotoxin (TTX) (Alomone Labs) were prepared in double distilled H_2O. Human kisspeptin-10 (Kp-10) (Calbiochem, EMD Biosciences, Inc) was diluted in DMSO (final concentration of DMSO in bath was 0.02%). All stock solutions were stored at −20°C. Kynurenic acid (Sigma Ltd) was freshly prepared in aCSF each experimental day. All drugs were applied in the perfusion solution with final concentration as indicated.

Analysis

Drug-induced changes in action potential (AP) frequency were determined as follows. 1) Each AP in the continuous-current recordings acquired at 10 kHz was detected by Clampfit 10, and the frequency of AP firing was determined by binning APs into 1-second bins. 2) Responses to drugs were determined by sorting all APs into 10-second bins and generating histograms of AP frequency. The control period was considered to be the 3-minute period immediately before drug administration. The response period was of variable duration (2–4 min) lasting from drug administration until the cell returned to baseline firing. The washout period was the 3-minute interval after the cell completed its response. 3) The percentage increase in firing during the response period was calculated as 100 × [(frequency in-drug − frequency of predrug)/frequency in-drug]. The percentage decrease in firing during the response period was calculated as 100 × [(frequency in predrug − frequency in-drug)/frequency predrug]. If a drug activated a silent neuron this was considered a 100% change. If a drug silenced a neuron, this was considered a 100% change. 4) And if the change in the average frequency of AP firing during the response period was more than 25% of the control period, the cell was considered to have responded to the drug. Statistical analysis using Kruskal-Wallis ANOVA was undertaken by comparing firing rates in control, response, and washout periods.

For whole-cell recordings, the amplitude of drug-induced outward/inward currents were determined as follows: recordings of continuous currents (holding potential = −60 mV) acquired at 1 kHz were filtered by low-pass 1-Hz filter (Gaussian) in Clampfit 10 with the root of mean square (RMS) of the filtered continuous currents being 0.33 ± 0.10 pA (mean ± SD, n = 46). If the difference between the average of 1-minute drug-induced peak current and 1-minute predrug current was more than 2× the SD of the predrug current, the difference was considered significant.

Statistical analysis was undertaken using Wilcoxon Signed Rank test for the comparison of firing rate before and during the test compound, Kruskal-Wallis ANOVA for comparison of groups, and Fisher's exact test for comparing the frequency of responses between groups.

Results

Kisspeptin modulation of GnRH neuron firing is dependent upon GPR54

Cell-attached recordings undertaken in brain slices from GnRH-GFP transgenic mice revealed potent stimulatory actions of 50nM, 100nM, and 400nM kisspeptin on 22 of 25 (88%) GnRH neurons (10 mice, 4 males, 6 diestrous females, age: 129 ± 25 d, 2–5 GnRH neurons recorded in each mouse) (Figure 1A). In contrast, recordings from 24 GnRH neurons obtained from 4 GnRH-GFP; GPR54KO mice (2 males, 2 females, age: 111 ± 15 d) and 4 GnRH-GFP;GnRH-Cre;GPR54$^{loxp/loxp}$ mice (2 males, 2 females, age: 123 ± 21 d) showed a complete absence of 100nM (Figure 1C) and 400nM (Figure 1B) kisspeptin action on GnRH neurons.

Kisspeptin modulates the firing of ARN neurons

Cell-attached recordings were made from phenotypically unidentified neurons located principally in the ventrolateral aspects of the ARN throughout its rostrocaudal length. Because a previous study reported effects of kisspeptin on ARN neurons at 1nM to 1μM concentrations (14), we began these studies using an intermediate kisspeptin concentration of 400nM. Recordings from 52 ARN neurons in GPR54-intact GnRH-GFP mice showed that 400nM kisspeptin exerted either stimulatory or inhibitory effects on the spontaneous firing rates of approximately one third of the ARN cells (22 of 52 neurons from 5 male and 11 diestrous female mice, age: 122 ± 11 d, 2–6 ARN neurons from each mouse). Of these responding cells, 9 (41%) significantly increased their firing rate by 48 ± 7% (0.43 ± 0.15 to 1.1 ± 0.52 Hz with recovery to 0.7 ± 0.33 Hz; $P < .05$, Kruskal-Wallis ANOVA) (Figure 2, A and B) and the other 13 (59%) neurons significantly decreased their firing rate by 76 ± 5% (0.40 ± 0.10 to 0.1 ± 0.03 Hz with recovery to 0.49 ± 0.2 Hz; $P < .01$, Kruskal-Wallis ANOVA) (Figure 2, C and D). Application of the vehicle DMSO had no effect on firing rates (0.50 ± 0.09 Hz [control period] vs 0.52 ± 0.10 Hz [DMSO]; n = 9; $P > .05$, Kruskal-Wallis ANOVA) (Figure 2, A and C). No differences were detected between males and females with 7/17 (41%) and 15/35 (43%) neurons responding to kisspeptin, respectively ($P = .85$; χ^2 test). Compared with the long-lasting actions of kisspeptin on GnRH neurons (Figure 1A), the effects of kisspeptin on ARN neuron firing were relatively short lived (Figure 2, A and C).

Kisspeptin modulation of ARN neuron firing is independent of GPR54

To assess the dependence of kisspeptin actions on GPR54 in the ARN, we undertook cell-attached recordings from 102 ARN neurons obtained from 17 male and 16 female GPR54KO or GnRH-GFP;GPR54KO mice

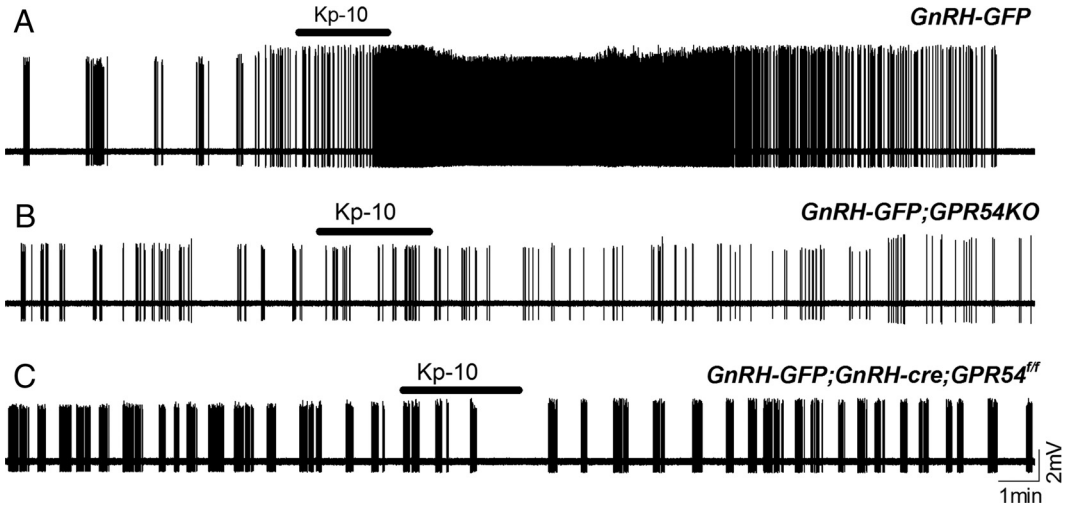

Figure 1. Kisspeptin (Kp-10) excites GnRH neurons through GPR54. Cell-attached voltage recordings of 3 GnRH neurons from a GnRH-GFP mouse (A), a GnRH-GFP;GPR54 knockout (GnRH-GFP;GPR54KO) mouse (B), and a GnRH-GFP;GnRH-cre;GPR54$^{flox/flox}$ (GPR54$^{f/f}$) mouse (C) showing the effect of a 2-minute 100nM (A and C) or 400nM (B) kisspeptin exposure.

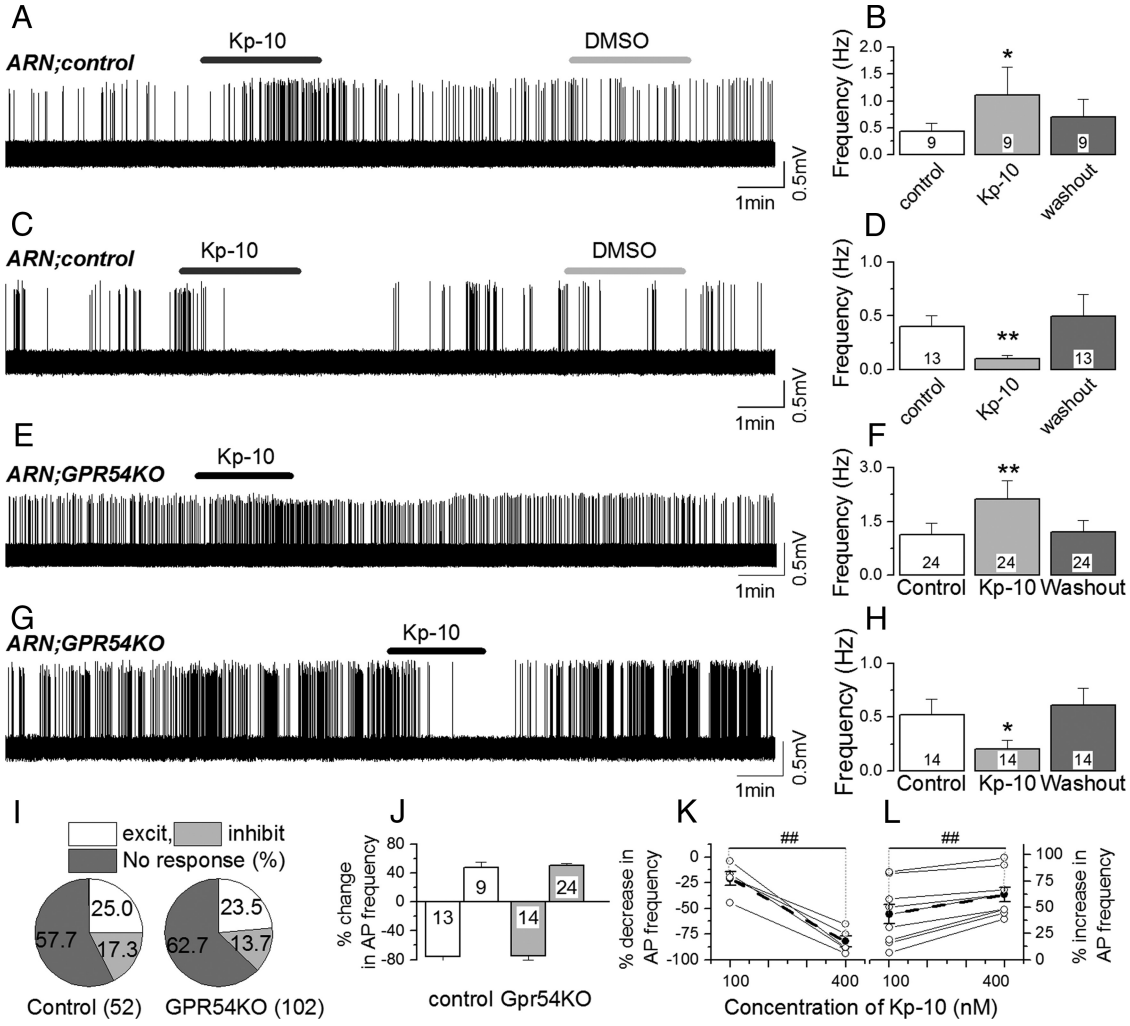

Figure 2. Kisspeptin (Kp-10) excites and inhibits arcuate neurons in both wild-type and GPR54 knockout mice. A and C, Cell-attached voltage recordings of 2 ARN neurons from a diestrous female GnRH-GFP mouse exhibiting increased (A) or decreased (C) firing in response to 400nM kisspeptin. B and D, Histograms summarizing all ARN neurons excited (A) or inhibited (C) by kisspeptin (400nM), respectively. E and G, Cell-attached voltage recordings of 2 ARN neurons from GPR54KO male (E) and GPR54KO female (G) mice showing that 400nM kisspeptin still excites (E) or inhibits (G) ARN neurons. F and H, Histograms summarizing all ARN neurons excited (E) or inhibited (G) by kisspeptin (400nM). I, Pie charts summarizing the percentage of ARN neurons excited (excit), inhibited (inhibit), or not affected (no response) by kisspeptin (400nM) from control and GPR54KO mice. Cell number given in parenthesis for both control and GPR54KO. J, Histograms summarizing the percentage increase and decrease in AP firing frequency induced by kisspeptin (400nM) from control and GPR54KO mice. Numbers of responding ARN neurons are given at the base of histograms. K, Plot showing the percentage decrease in firing rate evoked by 100nM and 400nM kisspeptin in individual ARN neurons (connected lines, n = 5) and mean response (dashed lines). L, Plot showing the percentage increase in firing rate evoked by 100nM and 400nM kisspeptin in individual ARN neurons (connected lines, n = 9) and mean response (dashed lines). ##, $P < .01$ compared with Kp-10 at 100nM, Wilcoxon Signed Ranks test. *, $P < .05$; **, $P < .01$, Kruskal-Wallis ANOVA.

(age, 110 ± 16 d; 2–6 ARN neurons from each mouse). In several cases, kisspeptin actions were assessed on both GnRH neurons and ARN cells from the same GnRH-GFP and GnRH-GFP;GPR54KO mice. All GPR54KO mice exhibited redundant testes or an absence of vaginal opening.

We found that, as in *Gpr54*-intact mice, approximately one third of ARN neurons (38 of 102) responded to 400nM kisspeptin in GPR54KO and GnRH-GFP; GPR54KO mice with no sex bias (males, 18 of 51 cells [35%]; females, 20/51 [39%]). Of the 38 neurons responding to kisspeptin, 24 (63%) exhibited a 50 ± 3% increase in firing (1.12 ± 0.33 to 2.12 ± 0.52 Hz with recovery to 1.20 ± 0.32 Hz; $P < .01$, Kruskal-Wallis ANOVA) (Figure 2, E and F), whereas the other 14 (37%) ARN neurons exhibited a 75 ± 5% decrease in firing (from 0.51 ± 0.12 to 0.19 ± 0.07 Hz with recovery to 0.60 ± 0.14 Hz; $P < .05$, Kruskal-Wallis ANOVA) (Figure 2, G and H). The percentages of ARN neurons excited or inhibited by kisspeptin (Figure 2I), and their percentage change in firing frequency (Figure 2J), were not different between *Gpr54*-intact and GPR54KO mice ($P > .05$, χ^2 test and Kruskal-Wallis ANOVA). These observations demonstrate the ability of kisspeptin to regulate the firing of ARN neurons in the absence of GPR54.

Because the kisspeptin responses are repeatable in the ARN, it was possible to examine the effects of both 100nM and 400nM kisspeptin on 14 kisspeptin-sensitive ARN neurons in wild-type (3 females and 5 males) and GPR54KO mice (4 females and 4 males) (age, 118 ± 15 d; 2–6 recordings from each mouse). With 100nM kisspeptin, 1 of 5 neurons were significantly ($P < .05$, Kruskal-Wallis ANOVA) inhibited (mean decrease of 21 ± 7% in 5 cells) (Figure 2K), and 5 of 9 cells significantly ($P < .05$, Kruskal-Wallis ANOVA) excited (mean increase of 43 ± 9% in 9 cells) (Figure 2L). For 400nM kisspeptin, all 5 cells were significantly inhibited (mean decrease of 82 ± 5%) (Figure 2K) and all 9 cells significantly excited (mean increase of 62 ± 7%) (Figure 2L). As a group, dose-dependent responses were observed for both the inhibitory ($P < .01$; paired-Wilcoxon Signed Rank test) (Figure 2K) and excitatory ($P < .01$; paired-Wilcoxon Signed Rank test) (Figure 2L) actions of kisspeptin on ARN cells. Because 400nM kisspeptin evoked larger changes in firing rate, this concentration was used for the remaining experiments.

Effects of kisspeptin on ARN neuron firing are mimicked by RFRP-3

Recent studies in cell expression systems indicate that kisspeptin can activate NPFF1 and NPFF2 receptors with high affinity (20–22), and both of these receptors are expressed by cells in the mouse ARN (23). Hence, one possible explanation for the GPR54-independent actions of kisspeptin on ARN neurons is that they are mediated by NPFFR.

To test this hypothesis, we first examined the effects of RFRP-3, an agonist of NPFF1 and NPFF2 receptors (22, 24), on ARN neurons that responded to kisspeptin. Eleven ARN neurons preidentified to be excited by 400nM kisspeptin were tested with 400nM RFRP-3. Nine of these 11 neurons were also found to be excited by RFRP-3 (Figure 3A) and exhibited the same dynamics of activation as that observed with kisspeptin; mean duration of activation 3.35 ± 0.2 minutes with a 55 ± 7% increase in firing (RFRP-3) compared with 2.77 ± 0.37-minute duration and 54 ± 8% increase in firing (kisspeptin) (Figure 3, A and B). The remaining 2 kisspeptin-activated cells did not respond to RFRP-3 (data not shown). Four out of 6 ARN neurons that were inhibited by kisspeptin were also found to be inhibited by RFRP-3 (Figure 3, C and D). The remaining 2 kisspeptin-inhibited cells did not respond to RFRP-3 (data not shown). This suggested that kisspeptin may be acting through NPFFRs in 76% (13/17) of ARN neurons.

RF9 is reported to be an antagonist at NPFFRs (25). As such, we used RF9 to try and assess whether kisspeptin actions in the ARN might be mediated by NPFFR. To our surprise, all 9 ARN neurons excited by kisspeptin and RFRP-3 were also found to be activated by RF9 alone (Figure 3, A and B). The RF9 excitation had a mean duration of response of 2.67 ± 0.15 minutes and generated a 56 ± 7% increase in firing that was the same as that observed with kisspeptin and RFRP-3 (Figure 3, A and B). Furthermore, all 4 ARN neurons inhibited by kisspeptin

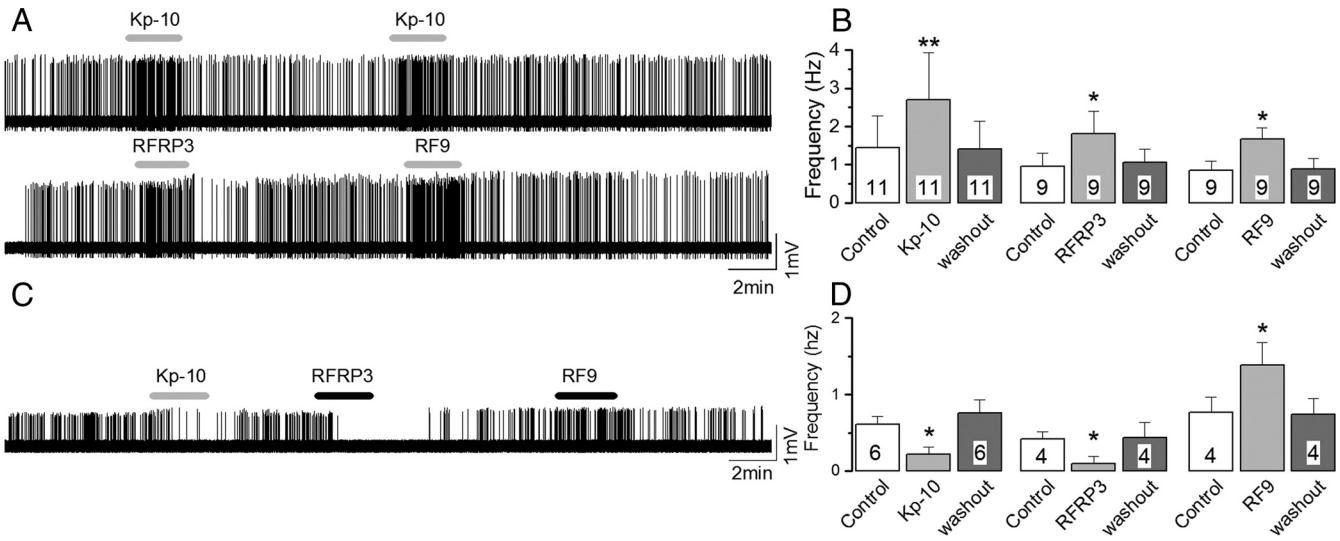

Figure 3. Effects of kisspeptin, RFRP-3, and RF9 on individual ARN neurons. A, Cell-attached voltage recording of an ARN neuron from a GPR54KO female mouse showing that kisspeptin (Kp-10, 400nM), RF9 (400nM), and RFRP-3 (400nM) all had the same excitatory action. B, Histograms summarizing all ARN neurons excited by kisspeptin (400nM), RF9 (400nM), and RFRP-3 (400nM). C, Cell-attached voltage recording of an ARN neuron from a GPR54KO male mouse showing that kisspeptin and RFRP-3 inhibited firing while RF9 continues to excite the cell. D, Histograms summarizing all ARN neurons inhibited by kisspeptin (400nM) and RFRP-3 (400nM) but excited by RF9 (400nM). Numbers of responding ARN neurons summarized are given at the base of histograms (B and D). *, $P < .05$; **, $P < .01$ comparing with control and washout, Wilcoxon Signed Ranks test.

and RFRP-3 were also excited by RF9 (Figure 3, C and D). Thus, RF9 was found to unexpectedly activate all kisspeptin and RFRP-3-sensitive ARN neurons regardless of whether kisspeptin/RFRP-3 was inhibitory or excitatory.

There is, however, a small population of ARN neurons (~24%) that respond to kisspeptin but not to RFRP-3 and are not activated directly by RF9. We persisted, therefore, in trying to assess the ability of RF9 to antagonize actions of kisspeptin in these cells where there would be no confounding stimulatory action. Seventeen such cells were identified (7 excited and 10 inhibited by kisspeptin) and treated with 400nM (n = 2), 1μM (n = 7), or 6μM (n = 10) RF9 before retesting with kisspeptin. RF9 was only able to suppress the inhibitory action of kisspeptin at 6μM (Figure 4, A–D) and had no significant effect at any concentration (1μM, n = 3; 6μM, n = 4) on kisspeptin's stimulatory actions (Figure 4, E and F).

We also tested whether BIBP-3226, another NPFF receptor antagonist that additionally has antagonistic activity at neuropeptide Y (NPY) Y1 receptors (26, 27), may suppress the effects of kisspeptin on ARN cells. Twelve out of 33 ARN neurons (from 6 GPR54KO mice, 3 female and 3 male, age: 75 ± 6 d) responded to kisspeptin by exhibiting reduced (n = 6) (Figure 5, A and B) or increased (n = 6) (Figure 5, C and D) firing in the presence of kynurenic acid, CNQX, and GABAzine. BIBP-3226 (1μM) alone had no effects on basal ARN neuron activity (Figure 5) but antagonized the kisspeptin responses of approximately half of ARN cells tested. BIBP-3226 antagonized the inhibitory responses of 4 out of 6 cells (Figure 5, A and E) and 2 of 6 cells excited by kisspeptin (Figure 5, C and G). The remaining cells remained inhibited (Figure 5, B and F) or excited (Figure 5, D and H) by kisspeptin in the presence of BIBP-3226.

Whole-cell recordings demonstrate direct actions of kisspeptin on ARN neurons

To examine whether kisspeptin acts directly on ARN neurons, whole-cell recordings were undertaken in the presence of kynurenic acid, CNQX, GABAzine, and TTX. Thirty-five (34%) of 103 arcuate neurons from 18 male and 8 female GnRH-GFP;GPR54KO mice (age, 99 ± 17 d; 3–6 ARN neurons from each mouse) recorded in whole-cell mode and held at −60 mV, responded to kiss-

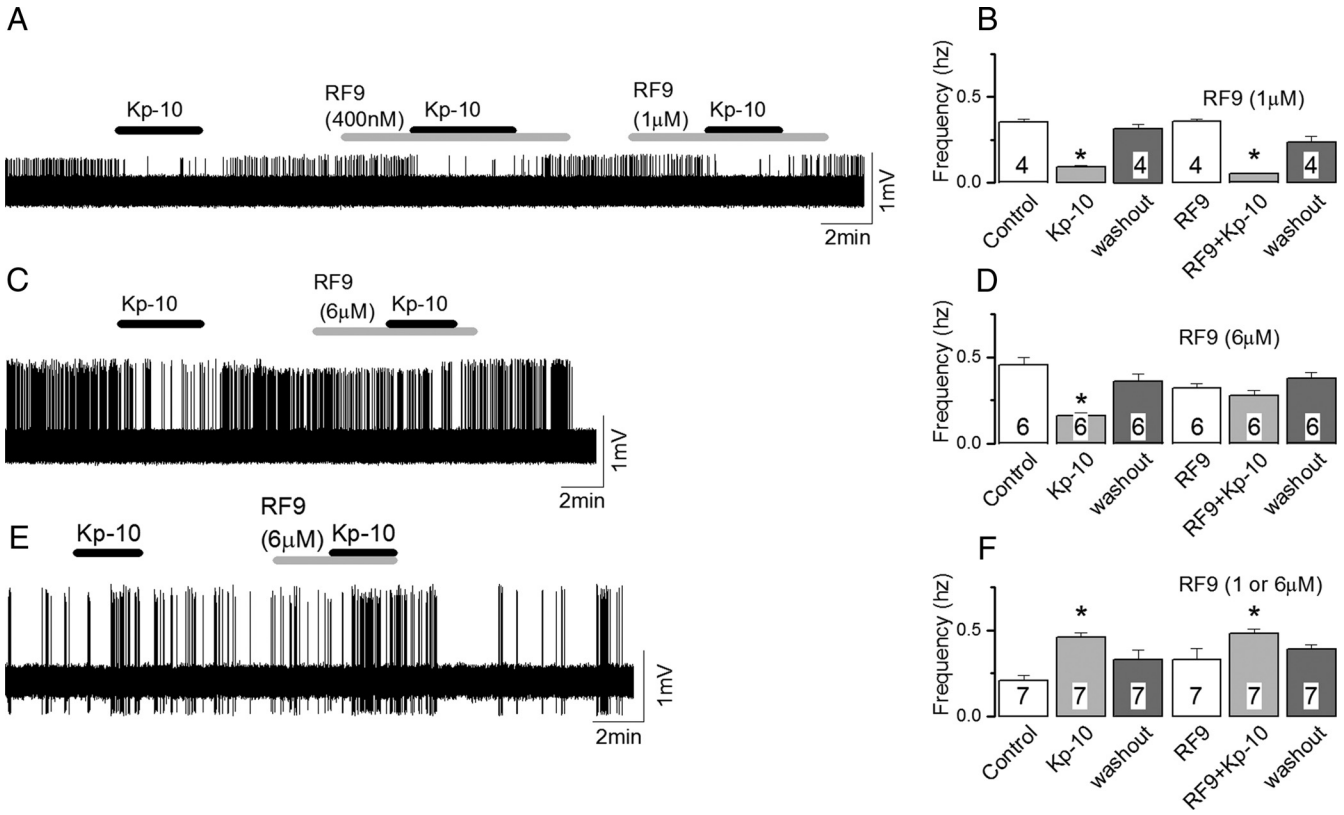

Figure 4. Kisspeptin (Kp-10) actions on ARN neurons can be blocked by RF9 only at high concentrations. A and C, Cell-attached voltage recordings from 2 ARN neurons from diestrous female mice showing that 400nM kisspeptin inhibitory actions are only blocked by the highest concentration (6μM) of RF9. B and D, Histograms summarizing the effects of 1μM (B) and 6μM (D) RF9 on the inhibitory actions of 400nM kisspeptin on ARN neurons. E, Cell-attached voltage recording from an ARN neuron from a male mouse showing that the high concentration (6μM) of RF9 does not affect 400nM kisspeptin excitatory actions. F, Histograms summarizing the effects of 1μM (n = 3) and 6μM RF9 (n = 4) on the excitatory actions of 400nM kisspeptin on ARN neurons. Numbers at the base of histograms indicate cell numbers. *, $P < .05$ comparing with control and washout, paired Wilcoxon Signed Ranks test.

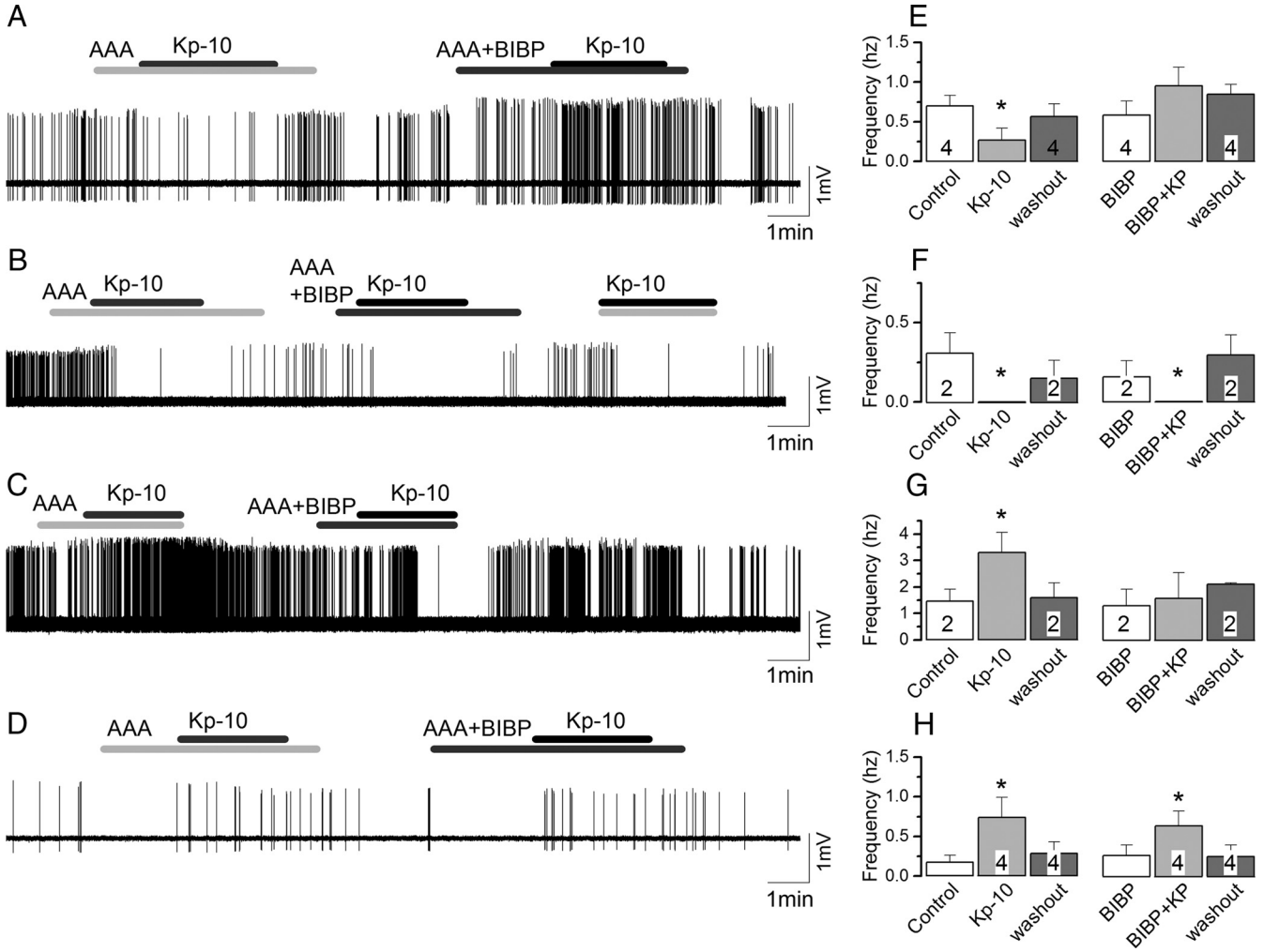

Figure 5. Kisspeptin (Kp-10) actions on ARN neurons can be blocked by BIBP-3226. A–D, Cell-attached voltage recordings of ARN neurons from adult male and female mice. The inhibitory effect of 400nM kisspeptin is either blocked (A) or unaffected (B) by 1μM BIBP-3226. The excitatory effect of 400nM kisspeptin is either blocked (C) or unaffected (D) by 1μM BIBP-3226 in the presence of the amino acid receptor antagonists (AAA) 2mM kynurenic acid, 20μM CNQX, and 5μM GABAzine. E–H, Histograms summarizing the effects of BIBP-3226 on the inhibitory (E and F) or excitatory (G and H) effects of kisspeptin. Numbers at the base of histograms indicate cell numbers. *, $P < .05$ compared with control and washout for individual cells (F and G) or the average of 4 cells (E and H), Kruskal-Wallis ANOVA.

peptin (400nM) with an inward (n = 19) (Figure 6A) or outward (n = 16) (Figure 6B) current. Peak inward currents were 15.1 ± 1.8 pA (range, 4.0–24.2 pA) with duration 3.8 ± 0.3 minutes (range, 2.0–7.0 min; n = 19), whereas peak outward currents were 7.4 ± 1.3 pA (range, 2.3–21.2 pA) with a duration of 3.7 ± 0.2 minutes (range, 2.2–5.0 min). The inward currents were accompanied by a decrease in input resistance (Figure 6, a1–a3) and a reversal potential of −22 ± 4 mV, indicating that kisspeptin likely opens nonselective cation channels to excite arcuate neurons. Outward currents were accompanied by a decrease in input resistance (Figure 6, b1–b3) and a reversal potential of −78 ± 6 mV, which is near the K^+ equilibrium potential of −85 mV, indicating that kisspeptin mainly opened K^+ channels to inhibit their excitability. The numbers of kisspeptin-sensitive ARN neurons exhibiting inward (54%) and outward (46%) currents are similar to the percentage of kisspeptin-sensitive ARN neurons excited (63%) and inhibited (37%) by kisspeptin in cell-attached studies. These data indicate that kisspeptin acts directly on ARN neurons to either excite or inhibit their activity.

Because RFRP-3 often replicated the effects of kisspeptin on ARN neuron firing rate, we next examined the effects of RFRP-3 under whole-cell conditions in GnRH-GFP;GPR54KO mice (from 13 male and 4 female GnRH-GFP;GPR54KO mice, age: 110 ± 12 d, 3–6 ARN neurons recorded from each mouse) in the presence of kynurenic acid, CNQX, GABAzine, and TTX. Twenty-two (34%) of 65 ARN neurons responded to 1μM RFRP-3 with 9 cells (41%) exhibiting an inward current with a peak current of 16.8 ± 3.1 pA (range, 5.3–26.5 pA) and duration 3.7 ± 0.3 minutes (range, 2.2–5 min) (Figure 6C), and 13 cells (59%) displaying an outward current with peak levels of 7.0 ± 1.5 pA (range, 2.3–19.4 pA) and duration of 3.8 ±

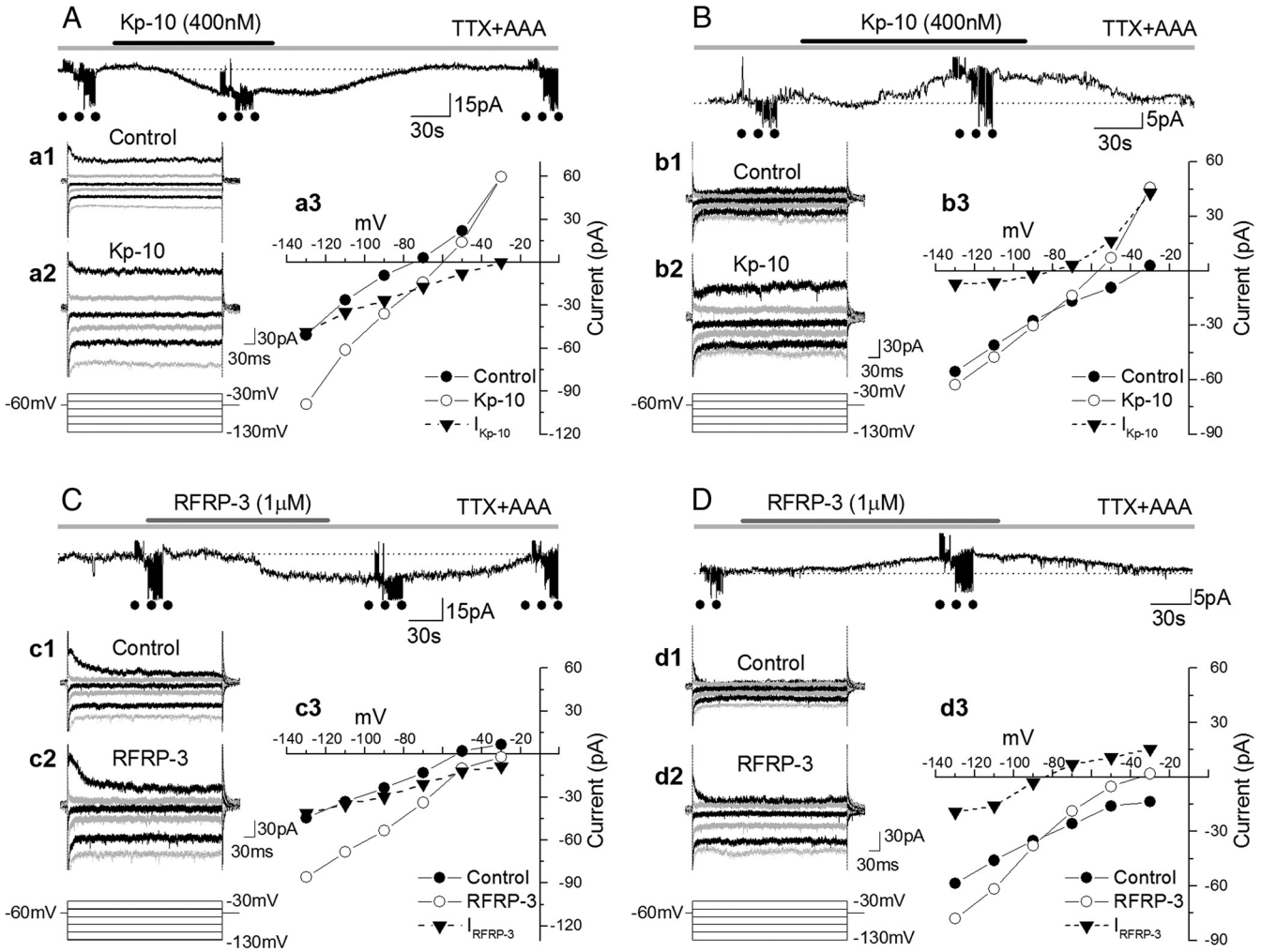

Figure 6. Kisspeptin (Kp-10) and RFRP-3 act directly on ARN neurons. A and B, Whole-cell current recordings of 2 ARN neurons from female (A) and male (B) GPR54KO mice showing that Kp-10 (400nM) induces inward (A) or outward (B) currents. C and D, Whole-cell current recordings of ARN neurons from male (C) and female (D) GPR54KO mice showing that RFRP-3 induces inward (C) or outward (D) currents. The black dots indicate the test voltage step-induced currents (the upward or downward deflections, truncated), and dotted lines indicate holding current levels and bars indicate the application of drugs. AAA, amino acid antagonists, including 2mM kynurenic acid, 20μM CNQX, and 5μM GABAzine. a1 and a2 show current responses in alternating black and gray lines to test voltage steps of −30, −50, −70, −90, and −110 mV during control (above) and drug (below). The protocol is shown below a2, where −60 mV is the holding potential. Values for each step were taken from the last 100 ms of recording. a3 shows current-voltage plots before (control, filled circles) and during Kp-10 application (Kp-10, open circles) taken from the cell shown in A. The Kp-10-induced current (I_{kp-10}, filled triangles) was obtained by subtraction of currents during control at each holding potential from the currents during Kp-10 at corresponding holding potentials. Traces (acquired at 20 kHz) in b1–b3, c1–c3, and d1–d3 represent the same protocols as detailed in a1–a3 above. The holding potential was −60 mV. The reversal potentials in a3, b3, c3, and d3 are −22, −80, −10, and −84 mV, respectively.

0.3 minutes (range, 2.2–5 min) (Figure 6D). The inward currents were accompanied by a decrease in input resistance (Figure 6, c1–c3) and a reversal potential of −23 ± 3 mV. Outward currents were accompanied with a decrease in input resistance (Figure 6, d1–d3) and a reversal potential of −86 ± 4 mV. Experiments undertaken in ARN cells from *Gpr54*-intact GnRH-GFP mice showed the same responses (Table 1). The overall responses of ARN neurons to kisspeptin and RFRP-3 were similar in both control and GPR54KO mice (Table 1) with the exception that the average peak amplitude ($P < .01$) and density ($P < .05$) of the kisspeptin-induced inward current being significantly larger in GPR54KO mice than that in control mice (Table 1). The average peak amplitudes and densities of kisspeptin-induced outward currents were the same in GPR54KO and control mice (Table 1).

These studies indicated that ARN neurons can respond in the same way to kisspeptin and RFRP-3 (Table 1). To address whether kisspeptin and RFRP-3 exert the same effects on the exact same cells, whole-cell recordings were made from 65 ARN neurons of which 27 responded to one or both peptides. Of these 27 ARN neurons, 14 (52%) cells exhibited activation of the same ion currents in response to both kisspeptin and RFRP-3 with 6 cells show-

Table 1. Summary of Actions of Kp-10 and RFRP-3 on ARN Neurons Recorded in Whole-Cell Mode

	Control mice		GPR54KO mice	
	Kp-10	RFRP-3	Kp-10	RFRP-3
Responding rate	33%	10%	34%	34%
Inward current (I_{inward}, pA)	4.1 ± 1.3 (4)[b]	5.4 ± 3.1 (2)	15.1 ± 1.8 (19)	16.8 ± 3.1 (9)
Density of I_{inward} (pA/pF)	0.24 ± 0.1 (4)[a]	0.27 ± 0.12 (2)	0.77 ± 0.13 (19)	0.80 ± 0.16 (9)
Membrane capacitance (pF)	16.7 ± 1.7 (4)	18.5 ± 3.0 (2)	22.0 ± 1.5 (19)	22.3 ± 1.9 (9)
Reversal of I_{inward} (mV)	−28 ± 6 (4)	−20 ± 8 (2)	−22 ± 4 (19)	−23 ± 3 (9)
Duration of I_{inward} (min)	3.8 ± 1.3 (4)	5.6 ± 1.0 (2)	3.8 ± 0.3 (19)	3.7 ± 0.3 (9)
Outward current ($I_{outward}$, pA)	4.8 ± 0.8 (9)	6.1 ± 2.9 (2)	7.4 ± 1.3 (16)	7.0 ± 1.5 (13)
Density of $I_{outward}$ (pA/pF)	0.34 ± 0.1 (9)	0.33 ± 0.19 (2)	0.46 ± 0.1 (16)	0.47 ± 0.13 (13)
Membrane capacitance (pF)	16.2 ± 2.1 (9)	19.5 ± 2.5 (2)	17.0 ± 1.6 (16)	17.1 ± 1.7 (13)
Reversal of $I_{outward}$ (mV)	−83 ± 3 (9)	−81 ± 6 (2)	−78 ± 6 (16)	−86 ± 4 (13)
Duration of $I_{outward}$ (min)	4.2 ± 0.6 (9)	3.7 ± 0.1 (2)	3.7 ± 0.2 (16)	3.8 ± 0.3 (13)

The cell number is given in parenthesis.

[a] $P < 0.05$ compared with GPR54KO mice.

[b] $P < 0.01$ compared with GPR54KO mice.

ing an inward current to both peptides (Figure 7A) and 8 cells showing an outward current to both peptides (Figure 7B). The I–V curves showed activation of very similar currents by kisspeptin and RFRP-3 in these cells (Figure 7, a1 and a2 and b1 and b2). The remaining 13 neurons displayed a response to either kisspeptin (5 cells) (Figure 7C) or RFRP-3 (8 cells).

Discussion

We report here that kisspeptin modulates neuronal activity in the absence of GPR54. As expected (3), kisspeptin actions on GnRH neurons were dependent completely upon GPR54 expression. However, within the ARN, approximately one third of all neurons responded to kisspeptin with either an increase or decrease in firing rate, and these responses were shown to be independent of GPR54. These effects of kisspeptin were relatively short lived (compared with kisspeptin actions on GnRH neurons), direct, repeatable, dose dependent, and shown to result from the likely activation of nonselective cation channels and/or closure of potassium channels. Importantly, one half to three quarters of kisspeptin-responsive ARN neurons were found to be activated in exactly the same manner by RFRP-3, suggesting that some kisspeptin actions in the ARN may come about through activation of NPFF receptors.

Kisspeptin regulation of ARN electrical activity

Fu and van den Pol (14) were the first to examine actions of kisspeptin in the mouse ARN and demonstrated that kisspeptin activated POMC neurons directly and NPY neurons indirectly. Recordings in the present study targeted the ventrolateral ARN, where POMC and NPY cells are located, but did not examine phenotypically identified ARN neurons. Nevertheless, we note that the excitatory responses recorded here are similar to those reported for POMC neurons (14). Fu and van den Pol reported that these actions on POMC neurons were likely to depend upon GPR54 as 1μM of peptide 234, an early GPR54 antagonist, was found to partially block the kisspeptin response (14). We show here that all of the excitatory and inhibitory actions of kisspeptin detected in a heterogeneous population of ARN neurons are independent of GPR54; the percentages of neurons exhibiting inhibitory and excitatory responses, and their degree of firing rate change are indistinguishable between wild-type and GPR54KO mice. Hence, although we cannot rule out the possibility that kisspeptin may activate POMC neurons through GPR54, the actions of kisspeptin on other neurons in the ARN are clearly independent of GPR54.

We note that the GPR54KO mouse model used in this study is hypogonadal with very low gonadal steroid levels (17). Although the percentage of ARN cells inhibited and excited by kisspeptin are very similar in intact and GPR54KO mice, we found that the inward (excitatory) current evoked by kisspeptin was significantly larger in GPR54KO mice compared with controls. This suggests that gonadal steroids do not influence the type of response an ARN cell exhibits to kisspeptin, but may regulate its magnitude. The size of kisspeptin-induced outward (inhibitory) currents were not, however, different between control and GPR54KO mice. As such, it is possible that gonadal steroids may selectively modulate nonselective cation channel expression (28) and/or their intracellular regulatory pathways to modify kisspeptin excitatory actions in the ARN.

What receptors may be mediating the actions of kisspeptin in the ARN?

The mammalian RFamide peptides share a common C-terminal Arg-Phe-amide motif and are classified into 5

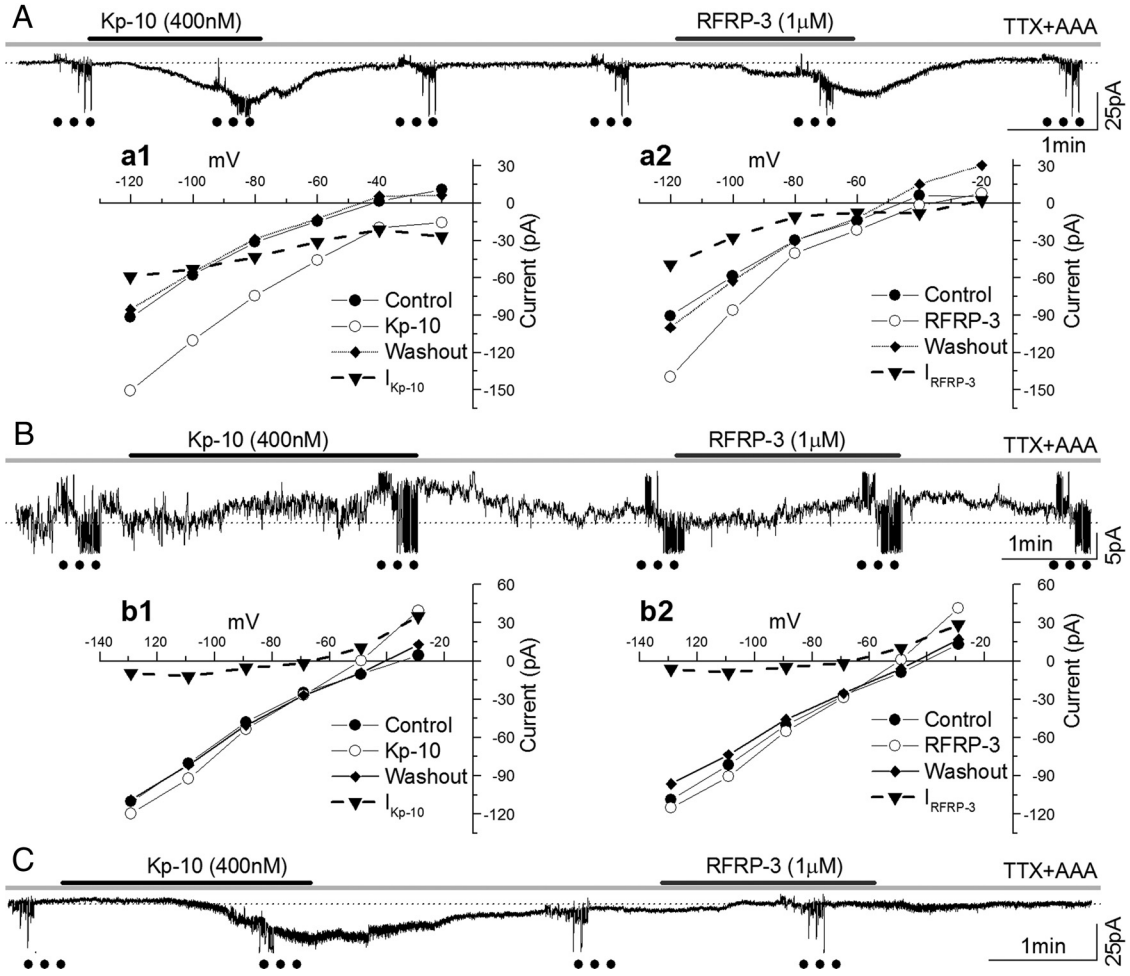

Figure 7. Kisspeptin (Kp-10) and RFRP-3 act directly on the same ARN neurons. A and B, Whole-cell current recordings of 2 ARN neurons from male (A) and female (B) GPR54KO mice showing that both Kp-10 (400nM) and RFRP-3 (1μM) induce inward (A) or outward (B) currents in the same cell. C, Whole-cell current recording of an ARN neuron from a female GPR54KO mouse showing that Kp-10 induces outward currents but RFRP-3 had no effect. The black dots indicate the test voltage step-induced currents (the upward or downward deflections, truncated), and dotted lines indicate holding current levels and bars indicate the application of drugs. AAA, amino acid antagonists including 2mM kynurenic acid, 20μM CNQX, and 5μM GABAzine. a1 shows current-voltage plots before (control, filled circles) and during Kp-10 application (Kp-10, open circles) and washout (filled diamonds) taken from the cell shown in A. Kp-10-induced current (I_{kp-10}, filled triangles) was obtained by the subtraction of currents during control at each holding potential from the currents during Kp-10 at corresponding holding potentials. b1 and b2 show the same information taken from the cell shown in B. Traces shown in a1 and a2 and b1 and b2 were acquired at 20 kHz. The voltage step protocol and the establishment of IV curves are the same as in Figure 6. The holding potential is −60 mV. The reversal potentials in a1, a2, b1, and b2 are 0, −23, −70, and −70 mV, respectively.

distinct families: RFRP-1/3, NPFF/AF, prolactin-releasing peptides (PrRPs), kisspeptins, and 26/43RFa (29, 30). Although each family has its own cognate receptor (respectively, NPFF1R, NPFF2R, GPR10, GPR54, and GPR103), there is increasing evidence for crosstalk signaling among the RFamide peptides (20, 22, 31). In relation to kisspeptin, recent studies that have expressed the different RFamide receptors in cell lines have shown that, in addition to GPR54, kisspeptin can bind to and activate NPFF1R and NPFF2R with high affinity (20–22). Recent in situ hybridization studies have shown the presence of NPFF1R and NPFF2R transcripts in ARN neurons (23). Thus, it seemed plausible that kisspeptin may act through NPFF receptors to activate and inhibit ARN neuron firing.

Our studies using the NPFF receptor agonist RFRP-3 (22, 32) suggest that kisspeptin may be acting through NPFF receptors to modulate the activity of subpopulations of ARN neurons. In cell-attached recordings from GPR54KO mice, 76% of neurons activated or inhibited by kisspeptin exhibited the same response to RFRP-3. In whole-cell recordings from GPR54KO mice, 52% of neurons tested with both peptides showed the same activation of ion channels to both peptides. Together, these observations lead us to suggest that kisspeptin may act through NPFF receptors to modulate the activity of ARN neurons. However, this may not be the case for all ARN neurons as the POMC cells were reported to be excited by kisspeptin but inhibited by RFRP-3 (14).

Because the ARN cells examined in this study are heterogeneous in phenotype, it is not surprising to find that kisspeptin/RFRP-3 can exert either stimulatory or inhibitory actions. Whether this represents 2 or more distinct populations of ARN neurons is unknown at this stage. Nevertheless, the direct activation of at least 2 different types of channels by RFRP-3 (and kisspeptin) in ARN cell populations indicates that diverse signaling pathways can be used by NPFF receptors in different ARN cell populations. To date, in vitro studies have identified that NPFF receptors are coupled to inhibitory Gi/o G protein-coupled receptors (21, 33) and this may account for the inhibitory actions reported here. Further studies will be required to determine the nature of the stimulatory effects of RFRP-3 on neurons. In this light it is interesting to note that GnRH neurons can exhibit both inhibitory and excitatory responses to RFRP-3 (19). However, we have found no evidence that any component of the stimulatory effects of kisspeptin on GnRH neurons are mediated by NPFF receptors as these cells fail to respond to 400nM kisspeptin in GnRH neuron-specific *GPR54*-null mice.

The RF9 compound was not found to act in the manner expected for an NPFF receptor antagonist in these studies. It is particularly hard to reconcile the observation that the NPFF receptor agonist RFRP-3 and antagonist RF9 exerted the same excitatory effects on the same ARN neurons. In this regard it may be pertinent to note that recent studies have shown that RF9 may, in fact, act as an agonist at NPFF receptors (34). Furthermore, in vivo investigations in the sheep and rodent have had difficulty reconciling the actions of RF9 as an NPFF receptor antagonist in the control of reproductive hormone secretion (35, 36). Indeed, RF9, at concentrations known to activate LH secretion ($>1\mu$M), has recently been shown to activate GnRH neurons directly in a GPR54-dependent manner (37). Thus, the pharmacological profile of RF9 is very unclear and, at the least, it appears not to be a pure NPFF receptor antagonist in the brain. Although we observed partial suppression of kisspeptin's inhibitory actions with the highest concentration of RF9 tested (6μM), it seems unsafe at present to ascribe this particular result to antagonism of NPFF receptors in the ARN.

Although BIBP-3226 has actions at the NPY Y1 receptor, it is also an NPFF receptor antagonist (26, 27). We found that BIBP-3226 effectively blocked excitatory and inhibitory actions of kisspeptin in half of ARN cells tested. Although this provides further evidence for kisspeptin acting through NPFF receptors within the ARN, the inability of BIBP-3226 to suppress all kisspeptin actions leaves open the possibility that other receptors may be involved in these responses. Other RFamide receptors that might conceivably play a role in kisspeptin's actions in the ARN could be GPR10, the PrRP receptor, and GPR103, the 26RFa/43RFa peptide receptor. However, there is little evidence that kisspeptin or RFRP-3 can bind to GPR10 in vitro (22) and, in the context of the present study, adult mouse GnRH neurons express NPFF1R (38, 39) and respond to RFRP-3 but not PrRP20 or PrRP31 (19). Similarly, there is little support at present for the possibility that kisspeptin may act through GPR103 (22).

Roles for kisspeptin in the ARN

A key question is whether the kisspeptin signaling reported here is physiological or pharmacological in nature. One of the remarkable features of kisspeptin's regulation of GnRH neurons is that, as a neuropeptide, it is extremely potent with even low 1nM–10nM concentrations having dramatic excitatory effects on cell firing (4, 8, 40). In contrast, we find here that 100nM–400nM concentrations of kisspeptin are required to modulate the electrical activity of ARN neurons in the acute brain slice. This is consistent with GPR54 not being the receptor activated in the ARN. More generally, we note that 100nM–400nM are themselves at the low range of neuropeptide concentrations normally required in brain slice experiments. For example, 300nM–1000nM concentrations of a variety of neuropeptides are required to modulate the firing of GnRH neurons (41). Hence, we believe that the concentrations of kisspeptin reported here to alter neuronal activity in the ARN are rather typical of neuropeptidergic regulation in the brain slice and likely reflect physiological modulation. It is kisspeptin-GPR54 coupling at the GnRH neuron that is unique.

Kisspeptin afferents to ARN neurons come from both the ARN and periventricular preoptic kisspeptin populations (42). The ability of kisspeptin to modulate the firing of up to one third of ventrolaterally positioned ARN neurons suggests that kisspeptin may have a role in regulating multiple homeostatic functions. Electrophysiological studies to date have shown that kisspeptin does not influence the firing of ARN kisspeptin neurons themselves (43) but can alter the excitability of POMC, GABA, and indirectly, NPY neuronal phenotypes in the ARN (14). In addition, there is evidence for kisspeptin fibers apposing dopaminergic neurons in the ARN (44, 45). Together, these data suggest that kisspeptin may have roles in regulating metabolic control (14, 15) as well as prolactin secretion (44, 45), and it will be of interest in future studies to establish the dependence of any such actions on GPR54 as opposed to NPFF receptors. Because most ARN neurons responsive to kisspeptin are also modulated by RFRP-3, it appears that kisspeptin and RFRP-3 afferents converge on ARN neurons to exert common excitatory or inhibitory effects upon their electrical activity. Whether the ARN

neurons targeted by kisspeptin and RFRP-3 are involved in networks regulating reproduction is unknown. It seems likely that the reported Gpr54-dependent"reproductive" actions of kisspeptin within the vicinity of the ARN (46, 47) result from the kisspeptin modulation of GPR54-expressing GnRH neuron dendrons (48, 49) adjacent to the median eminence.

In summary, we report here that kisspeptin can have actions in the brain that are independent of GPR54. The neuroanatomical mismatch between GPR54 and kisspeptin peptide expression in the mouse brain (10, 12, 50) had always suggested that there may be other ligands for GPR54 and other receptors for kisspeptin. The ARN, with probably the highest density of kisspeptin fibers in the brain, is one brain region where kisspeptin acts through receptors other than GPR54 to modulate neuronal activity. The identity of the "kisspeptin receptor" in the ARN is not entirely clear but evidence is provided to suggest that it may at least partly involve NPFF receptors. Future studies with NPFF receptor mutant mice (51) and the development of highly selective NPFF antagonists will be required to make further progress this field.

Acknowledgments

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