

Direct Regulation of GnRH Neuron Excitability by Arcuate Nucleus POMC and NPY Neuron Neuropeptides in Female Mice

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Hypothalamic neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons act to sense and coordinate the brain's responses to metabolic cues. One neuronal network that is very sensitive to metabolic status is that controlling fertility. In this study, we investigated the impact of neuropeptides released by NPY and POMC neurons on the cellular excitability of GnRH neurons, the final output cells of the brain controlling fertility. The majority (~70%) of GnRH neurons were activated by α -melanocyte-stimulating hormone, and this resulted from the direct postsynaptic activation of melanocortin receptor 3 and melanocortin receptor 4. A small population of GnRH neurons (~15%) was excited by cocaine and amphetamine-regulated transcript or inhibited by β -endorphin. Agouti-related peptide, released by NPY neurons, was found to have variable inhibitory (~10%) and stimulatory (~25%) effects upon subpopulations of GnRH neurons. A variety of NPY and pancreatic polypeptide analogs was used to examine potential NPY interactions with GnRH neurons. Although porcine NPY (Y1/Y2/Y5 agonist) directly inhibited the firing of approximately 45% of GnRH neurons, [Leu³¹,Pro³⁴]-NPY (Y1/Y4/Y5 agonist) could excite (56%) or inhibit (19%). Experiments with further agonists indicated that Y1 receptors were responsible for suppressing GnRH neuron activity, whereas postsynaptic Y4 receptors were stimulatory. These results show that the activity of GnRH neurons is regulated in a complex manner by neuropeptides released by POMC and NPY neurons. This provides a direct route through which different metabolic cues can regulate fertility. (*Endocrinology* 153: 5587–5599, 2012)

The integration of metabolic cues by the neuronal network controlling fertility is critical for the appropriate timing of puberty and reproduction in mammals (1, 2). States of under- or overnutrition result in disordered fertility in mice and humans (3, 4). Circulating leptin concentrations, reflecting fat stores, provide one important cue to the fertility network and act as a permissive regulator of puberty onset and ovarian cyclicity (1). Equally, insulin is also emerging as an important peripheral signal that may impact upon reproductive status (5).

The GnRH neurons represent the final output cell of the neuronal network controlling fertility in all mammals, and

investigators are presently unraveling the mechanisms and pathways through which leptin and insulin modulate the activity of these cells. Evidence now indicates that neither leptin nor insulin act directly upon GnRH neurons to regulate fertility (6–8). Instead, these hormones likely modulate the activity of multiple different afferent inputs to GnRH neurons. Recent work has demonstrated that neurons located in the ventral premammillary nucleus play an important role in mediating the actions of leptin on GnRH neurons (9–11). More generally, the proopiomelanocortin (POMC)/cocaine and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY)/agouti-related

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Abbreviations: aCSF, Artificial cerebrospinal fluid; AgRP, agouti-related peptide; ARN, arcuate nucleus; CART, cocaine and amphetamine-regulated transcript; GFP, green fluorescent protein; hPP, human PP; MC3R, melanocortin receptor 3; MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; pNPY, porcine NPY; POMC, proopiomelanocortin; rPP, rat pancreatic polypeptide; TTX, tetrodotoxin.

peptide (AgRP) neurons of the arcuate nucleus (ARN) are established as important mediators of leptin and insulin actions on multiple neuronal networks, including that regulating food intake (12). Although the involvement of these ARN neurons in regulating fertility is attractive, in that it would enable the coordinated control of feeding and reproduction by leptin and insulin, evidence is controversial. Early studies examining mice with POMC-specific deletions of the leptin receptor found no reproductive phenotype (13, 14), whereas more recent investigations using mutant mice with both individual and coupled leptin and insulin receptor deletions have revealed substantial reproductive deficits (15).

An essential piece of evidence supporting a role for POMC and NPY neurons in regulating GnRH neurons would be the demonstration that they can modulate GnRH neuron excitability directly. Present evidence exists for direct inputs from uncharacterized ARN neurons to GnRH cells in the mouse (16), and both NPY and POMC terminals can be identified synapsing on GnRH neurons (17, 18). However, to date, there has only been a single study examining the effects of NPY on adult GnRH neuron excitability (19). In the present series of studies, we have investigated the effects of a range of neuropeptides released by POMC and NPY neurons on adult female mouse GnRH neurons and characterized the receptor subtypes mediating their different actions.

Materials and Methods

Experimental animals

Adult female GnRH-green fluorescent protein (GFP) mice (20) were housed under constant conditions of light (12 h of light, from 0700 h) and temperature (22 C) with *ad libitum* access to food and water. Diestrous female mice were used for all experiments with the estrous cycle stage determined by daily vaginal smear. Experimental procedures were approved by the University of Otago Animal Welfare and Ethics Committee.

Brain slice preparation and electrophysiology

Animals were killed by cervical dislocation between 1000 and 1130 h, and brain was dissected and cut into 200-μm-thick sagittal slices with a vibratome (Leica VT1000S; Leica, Heerbrugg, Switzerland). During the slicing procedure, the brain was immersed in cold (~4 C), oxygenated artificial cerebrospinal fluid (aCSF) containing: 118 mM NaCl, 3 mM KCl, 11 mM D-glucose, 10 mM HEPES, 25 mM NaHCO₃, 0.5 mM CaCl₂, and 6 mM MgCl₂. Brain slices were transferred to a slice chamber (heated at 30 ± 2 C) containing oxygenated “recording aCSF”: 118 mM NaCl, 3 mM KCl, 11 mM D-glucose, 10 mM HEPES, 25 mM NaHCO₃, 2.5 mM CaCl₂, and mm 1.2 MgCl₂ (pH 7.3) for at least 1 h before recording. Loose-patch, whole-cell, or perforated-patch recordings were undertaken in slices placed in a recording chamber perfused with aCSF at 2–3 ml/min, maintained at 32 ± 1 C, and mounted in a fixed-stage upright fluorescence microscope (BX51WI; Olympus, Tokyo, Japan). GnRH-GFP neurons were identified briefly using fluorescence and then patched under differential interference contrast microscopy (using a ×40 water-immers-

TABLE 1. Effects of POMC and NPY neuron-released neuropeptides and agonists on GnRH neurons

Treatment	Receptor affinity	Dose	Total no.	Activation	Inhibition	No response
POMC neuron-released neuropeptides and agonists						
α-MSH		1 μM	(n = 13)	9	0	4
CART		1 μM	(n = 8)	2	0	6
		100 nM	(n = 5)	0	0	5
β-Endorphin		0.5 μM	(n = 11)	0	2	9
DAMGO		10 μM	(n = 3)	0	0	3
		1 μM	(n = 2)	0	0	2
MCR4 agonist	MC4R	10 nM	(n = 6)	4	0	2
D-Trp-γ-MSH	MC3R	10 nM	(n = 6)	4	0	2
NPY neuron-released neuropeptides and agonists						
pNPY	Y1/Y2/Y5	300 nM	(n = 13)	0	6	7
		100 nM	(n = 4)	0	0	4
NPY Y5 agonist	Y5	300 nM	(n = 4)	0	0	4
		100 nM	(n = 5)	0	0	5
		10 nM	(n = 4)	0	0	4
(Leu ³¹ ,Pro ³⁴)-NPY	Y1/Y4/Y5	300 nM	(n = 6)	3	2	1
		100 nM	(n = 8)	3	0	5
rPP	Y4	300 nM	(n = 10)	3	0	7
		30 nM	(n = 11)	3	0	8
AgRP	MC3R/MC4R	100 nM	(n = 11)	3	1	7
		10 nM	(n = 13)	3	1	9

Upper panel, No. of GnRH neurons displaying activation, inhibition, or no response to selected neuropeptides released by POMC neurons and specific agonists in loose-patch configuration. Treatments that failed to elicit responses to low dose were assessed at a higher (10-fold) dose. Lower panel, No. of GnRH neurons showing activation, inhibition, or no response to selected NPY agonist receptors and AgRP at different concentrations in loose-patch mode. Principal receptor affinity for each drug is also indicated in the second column. Data on selectivities of NPY analogues at different NPY receptors compiled from Refs. 83–86. NPY Y5 agonist, [cPP1-7, NPY19-23, Ala³¹, Aib³², Gln³⁴]-hPP. MCR4 agonist, Cyclo (-Ala-His-D-Phe-Arg-Trp-Glu)-NH₂.

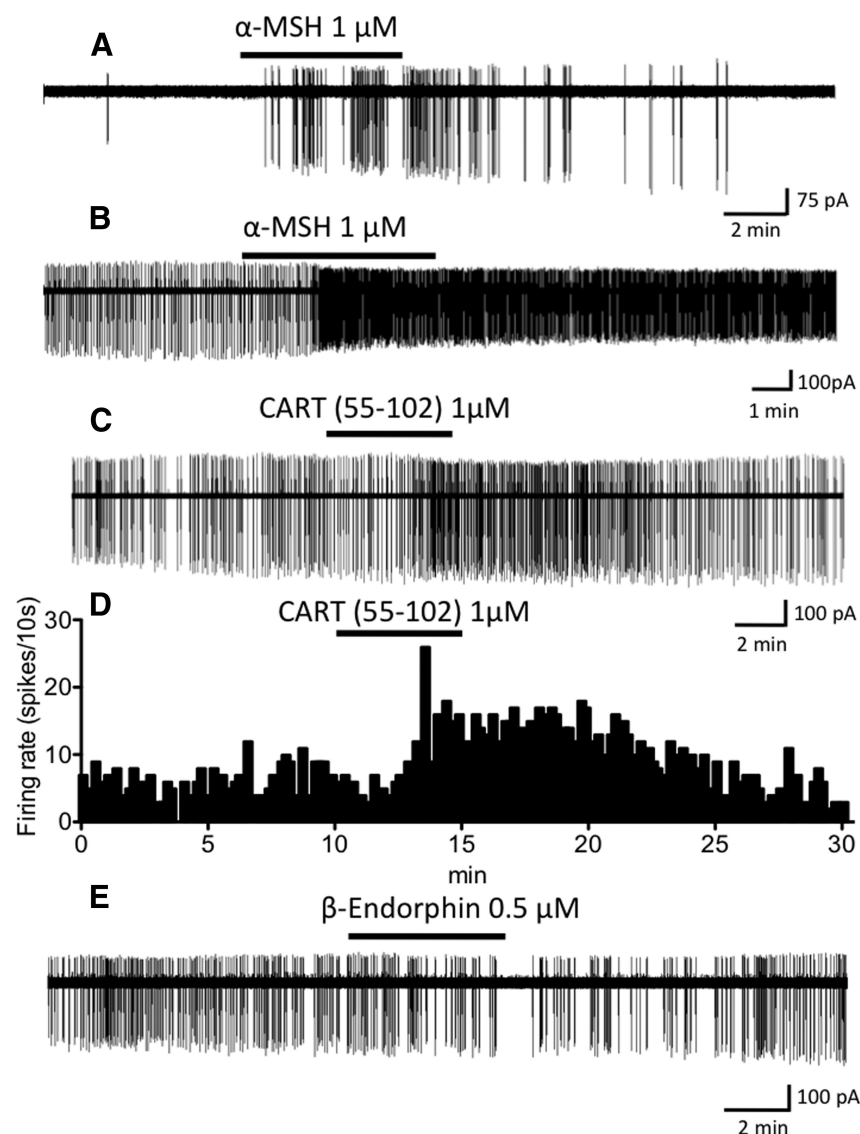


FIG. 1. Effects of neuropeptides released by POMC neurons on GnRH firing. Voltage-clamp recordings were made from GnRH neurons in loose-patch configuration. α -MSH (A and B), and to a lesser extent CART (C), application to the bath solution for a 5-min period increased GnRH cell firing, whereas β -endorphin (E) marginally decreased activity. D, Frequency-time histogram of firing rate displayed by the cell shown in C.

sion objective). Recording electrodes were pulled from borosilicate glass capillaries with a microelectrode puller (model P-97; Sutter Instruments, Navato, CA). Electrodes (resistances 3–5 M Ω) for loose-patch recording were filled with the following solution: 135 mM K-gluconate (D-gluconic acid K salt), 5 mM NaCl, 10 mM HEPES, 10 mM EGTA, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM MgATP, and 0.1 mM Na₂GTP (pH 7.32 adjusted with 1 M KOH). For whole-cell recording, the pipette solution contained: 135 mM K-gluconate (D-gluconic acid K salt), 5 mM NaCl, 10 mM HEPES, 1 mM BAPTA, 0.22 mM CaCl₂, 0.2 mM Na₂ATP, 2 mM MgATP, 0.2 mM Na₂GTP, and 7 mM phosphocreatine-Tris (pH 7.32). Perforated-patch recording was undertaken as described previously (21). In brief, gramicidin 10 mg/ml (Sigma Ltd., Auckland, New Zealand), dissolved in dimethylsulfoxide, was diluted to a final concentration of 30 μ g/ml in whole-cell pipette solution just before use and sonicated for 3 min. Then, GnRH-GFP neurons were patched to gigaohm

seal, and access resistance was monitored until 50–90 M Ω was reached (~30–40 min). Voltage and current signals were obtained using a Multiclamp 700B amplifier (CV7B; Molecular Devices, Foster City, CA) and sampled online with the use of a Digidata 1440 A interface (Molecular Devices) connected to a personal computer. Acquisition and subsequent analysis of the acquired data were performed using the Clampex 10 suite of software (Molecular Devices).

For loose-patch experiments, neurons were recorded for at least 15–20 min before drug application to ensure the stability of the recording. Drugs were applied for 5 min followed by 10–15 min of wash-out period. In whole-cell and perforated-patch configuration, drugs were applied for a 3- to 4-min period. In whole-cell and perforated-patch experiments, liquid junction potential was not corrected.

Analysis

In loose-patch experiments, cells were considered to have responded using the following criteria: the mean action current frequency was calculated during the 5-min period before drug exposure and during the last 4 min of drug application. If the mean frequency during the treatment was higher or lower than that of the control period plus twice the SD, the cell was considered to have been excited or inhibited, respectively.

In whole-cell and perforated-patch experiments, effects of drugs on membrane potential were assessed by determining the mean membrane potential 1 min before drug exposure and 1 min corresponding to the maximal response within the treatment period. If the average of the 1-min treatment was more than the average of the 1-min pretreatment period plus two times the SD, the difference was considered significant. To perform this analysis in cells showing spontaneous activity,

filtering of action potentials was achieved by sampling at 4 kHz and filtering with a Gaussian low-pass filtering (–3-dB cutoff at 0.8 Hz) in Clampfit.

Drugs

The following neuropeptides were used. For POMC neurons; α -melanocyte-stimulating hormone (MSH), mouse β -endorphin, and rat CART 55–102 (all from Bachem, Bubendorf, Switzerland), melanocortin receptor 3 (MC3R) agonist, D-Trp- γ MSH (American Peptide Co., Inc., Sunnyvale, CA) and MC4R agonist, Cyclo (β -Ala-His-D-Phe-Arg-Trp-Glu)-NH₂ (Phoenix Pharmaceuticals, Inc., Burlingame, CA), the μ -opioid receptor-specific agonist (D-Ala²), N-Me-Phe⁴, glycylol⁵-Enkephalin (DAMGO) (Bachem). For NPY neurons, mouse AgRP (82–131)-amide (Phoenix Pharmaceuticals, Inc.) porcine NPY (pNPY)

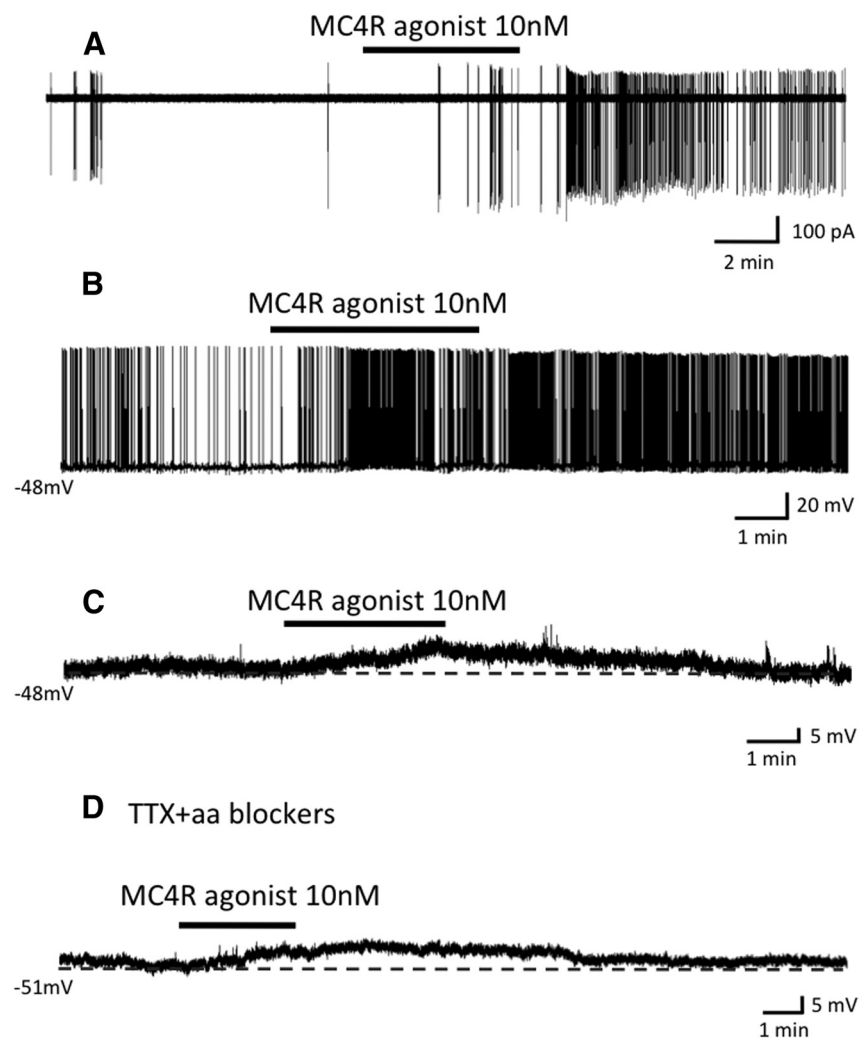


FIG. 2. MC4R agonist excites GnRH neurons. A, Representative recording, in loose-patch mode, of the long-lasting activation produced by MC4R agonist on GnRH neurons. The same effect was confirmed in whole-cell configuration (B and C). The increase in frequency of action potentials was accompanied by a modest depolarization (B), although this depolarization did not evoke action potentials in some cells (C). Depolarization produced by MC4R agonist persists in presence of TTX and amino acid (aa) blockers (D).

and rat pancreatic polypeptide (rPP) (from Bachem). [Leu³¹, Pro³⁴]-NPY (human, rat) and [cPP1-7, NPY19-23, AL³¹, Aib³², Gln³⁴]-human PP (hPP) were from Tocris Bioscience (Ellisville, MO). In some cells, 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione, 20 μ M DL-2-amino-5-phosphonopentanoic acid, picrotoxin 100 μ M, and 0.5 μ M tetrodotoxin (TTX) were included in the perfused aCSF solution during the recording to block presynaptic transmission. All the drugs were prepared in double distilled H₂O (except picrotoxin, in dimethylsulfoxide) at 10³ times final concentration, stored at –20 C, and diluted in aCSF just before use.

Results

In all cases, the numbers of GnRH neurons tested are annotated using “n,” whereas the numbers of animals used are given as “N” in parentheses.

Effects of neuropeptides released by POMC neurons on GnRH neurons

α -MSH and CART stimulate, whereas β -endorphin reduces, GnRH neuronal activity

The effects of POMC-derived neuropeptides (α -MSH and β -endorphin) and CART were initially assessed on GnRH neurons using loose-patch recordings (results summarized in Table 1, upper panel).

Application of α -MSH (1 μ M) (based on previous publications, see Refs. 22, 23) for 5 min produced an increase in the firing rate of nine of 13 (69%) GnRH neurons (N = 6) (Fig. 1, A and B). Two responding cells were activated from relative silence in the pretest period (Fig. 1A), and the remaining seven spontaneously active cells exhibited a robust 2- to 50-fold increase in firing rate (Fig. 1B). Four cells did not respond. For all experiments, cells that were silent and did not respond to drugs were tested with 20 mM KCl at the end of the experiment and only included in the analysis if they responded to this challenge.

CART was tested at 100 nM and 1 μ M (24). Exposure of five GnRH neurons (N = 4) to 100 nM CART had no effect on cell firing. At 1 μ M, two of eight (25%) GnRH neurons (n = 6) exhibited a modest, approximately 2-fold, increase in firing rate (Fig. 1, C and D).

β -Endorphin at 0.5 μ M (25) decreased the firing rate of two of 11 GnRH neurons (n = 7) by approximately 30% (Fig. 1E). The remaining cells were unaffected. To test this response further, five GnRH neurons (N = 4) were tested with 1 or 10 μ M DAMGO (26), the μ -opioid receptor agonist, but none responded.

α -MSH excites GnRH neurons through both MC3R and MC4R

The above studies showed that, of the POMC neuron-secreted peptides, α -MSH exerted the most potent actions. Because MC3R and MC4R are the only melanocortin receptors expressed at appreciable levels in the brain (27, 28), we used specific agonists at these receptors to examine further the α -MSH actions on GnRH neurons.

In loose-patch recordings, 10 nM Cyclo (β -Ala-His-D-Phe-Arg-Trp-Glu)-NH₂, the MC4R agonist (29),

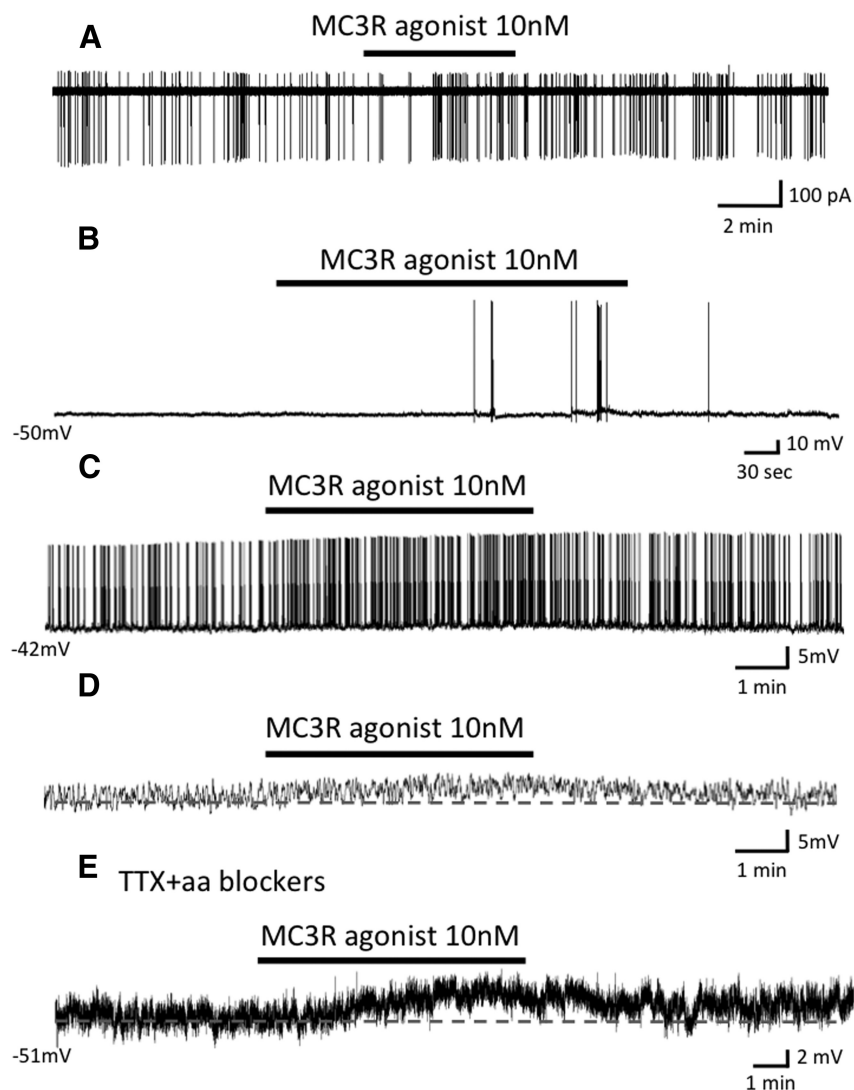


FIG. 3. MC3R agonist excites GnRH neurons. A, Representative loose-patch recording from a GnRH neuron excited by MC3R agonist. B, Current-clamp recording in whole-cell mode shows GnRH activation in response to MC3R agonist that was not accompanied by depolarization. In perforated-patch configuration (which keeps intact the intracellular milieu), MC3R increases GnRH cell firing, and unlike whole-cell mode, a small depolarization is detected (C and D). D, Membrane potential from the cell above after removing action potentials by Gaussian low-pass filtering (–3-dB cutoff at 0.8 Hz) in Clampfit (87). Also, in perforated-patch, the depolarization caused by MC3R treatment is maintained in presence of TTX and amino acid (aa) blockers (E).

elicited a potent and typically long-lasting increase in firing rate from four of six (66%) GnRH neurons recorded ($N = 3$) (Fig. 2A). Voltage recordings in the whole-cell configuration showed a very similar long-lasting increase in the frequency of action potentials (Fig. 2B) in eight of 11 (72%) GnRH neurons ($N = 5$). The increase in firing frequency was accompanied by a small membrane depolarization (2.9 ± 0.5 mV; $N = 8$), although in two cases, an increase in membrane potential did not evoke action potential firing (Fig. 2C). When synaptic transmission was blocked using a cocktail of TTX and amino acid receptor blockers (6-cyano-7-ni-

troquinoxaline-2, 3-dione, 2-amino-5-phosphonopentanoic acid, and picrotoxin), the MC4R agonist was still able to depolarize three of five GnRH neurons (3.2 ± 0.71 mV) (Fig. 2D).

In loose-patch recordings, the MC3R agonist DTrp- γ MSH at 10 nM (30) was also found to evoke a 1.5- to 2-fold increase in firing frequency in four of six (66%) GnRH neurons ($n = 5$) (Fig. 3A). This response was more modest than that found with the MC4R agonist. Experiments undertaken in whole-cell mode also revealed an increase in firing rate in two out of four (50%) GnRH neurons, although in this case, no change in membrane potential was observed (Fig. 3B). Because the dialysis of the recorded cell by the contents of the patch pipette unavoidably alters its intracellular milieu, we tested the effects of the MC3R agonist using the perforated-patch approach that avoids this problem. Under these conditions, the MC3R agonist continued to increase the firing rate of GnRH neurons but was now also found to evoke a small increase in membrane potential (2.6 ± 0.3 mV) in three of four GnRH neurons ($N = 4$) (Fig. 3, C and D). Using the same perforated-patch approach, but with inclusion of TTX and the amino acid receptor blockers, MC3R continued to depolarize three out of five GnRH neurons (2.3 ± 0.1 mV) (Fig. 3E).

Effects of neuropeptides released by arcuate NPY neurons on GnRH neurons

To study the possible influence of arcuate NPY neurons on GnRH neuronal activity, we assessed the effects of different NPY receptors agonists and AgRP on GnRH neurons. Given the complexity of NPY pharmacology and the lack of pure agonists for each NPY receptor, we used a selection of mixed and highly selective agonists to evaluate the possible influence of each receptor (results summarized in Table 1, lower panel).

pNPY (Y1/Y2/Y5 receptor agonist) inhibits GnRH neurons

GnRH neurons were tested initially with 100 and 300 nM (19, 31) pNPY using loose-patch recordings. Exposure to

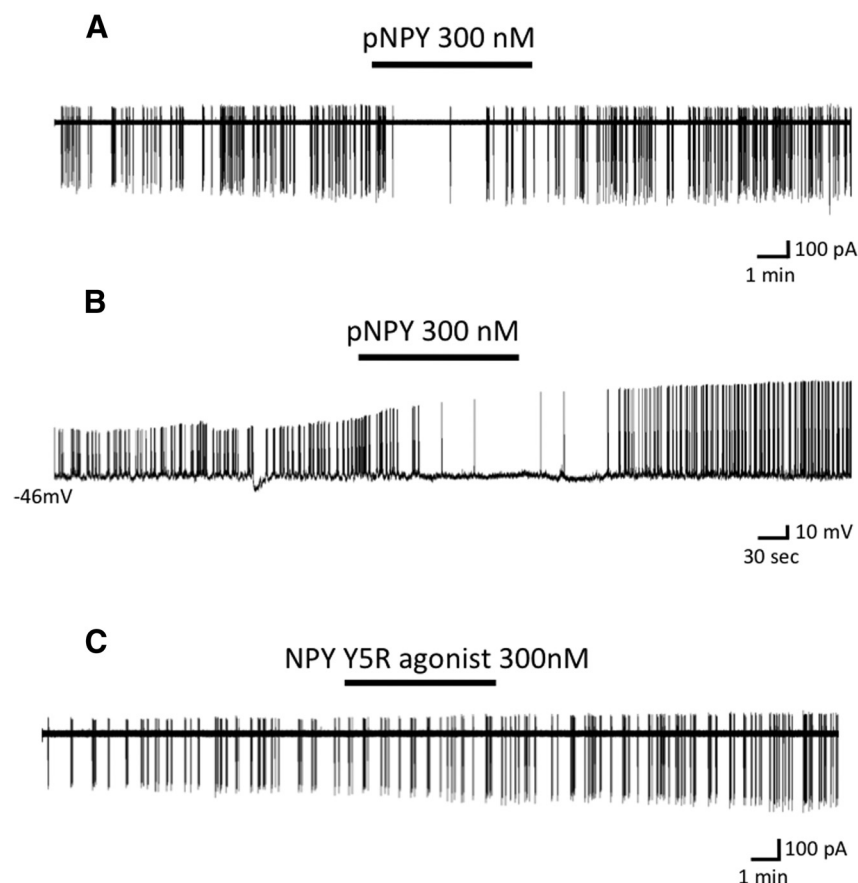


FIG. 4. Y1/Y2/Y5 receptor agonist pNPY inhibits, whereas Y5R agonist has no effect on GnRH neuron activity. A, Representative recording, in loose-patch mode, showing the significant inhibitory effect produced by pNPY, which was also confirmed in perforated-patch configuration (B). C, Y5R agonist does not affect GnRH neuron firing.

100 nM pNPY had no effect on GnRH neuron firing ($N = 4$). However, treatment with 300 nM pNPY resulted in a robust $66 \pm 13\%$ suppression of firing rate in six of 13 (46%) GnRH neurons ($N = 5$) (Fig. 4A and Table 1, lower panel). Experiments using whole-cell and perforated-patch recording modes found the same effects of pNPY with two of five GnRH (40%) neurons ($N = 3$) exhibiting a suppression of firing in response to 300 nM pNPY (Fig. 4B). There was no effect of pNPY on membrane potential in any of eight GnRH neurons tested in the absence of TTX and amino acid blockers.

A NPY Y5 receptor agonist has no effect on GnRH neuron firing rate

The above observation suggested that activation of Y1, Y2, and/or Y5 receptors inhibits GnRH neuron excitability. To define the receptors involved in this action, we examined the effect of [cPP1-7, NPY19-23, AL³¹, Aib³², Gln³⁴]-hPP, a highly selective Y5 receptor agonist (32). Thirteen GnRH neurons ($n = 9$) tested with 10, 100, and 300 nM concentrations of the Y5 agonist did not show any

response in loose-cell patch (Fig. 4C and Table 1, lower panel).

A Y1/Y4/Y5 receptor agonist [Leu³¹,Pro³⁴]-NPY excites and inhibits GnRH neurons

The above two experiments indicated that Y1 and Y2 receptors were likely to be responsible for suppressing GnRH neuron firing rate. To differentiate this mechanism further, we tested the effects of [Leu³¹,Pro³⁴]-NPY that has been used previously as a probe for Y1 actions on GnRH/LH release (33–35). Despite this, it is noteworthy that [Leu³¹,Pro³⁴]-NPY also displays high affinity for Y4 and Y5 receptors (36). Because the Y5 receptor is not relevant for GnRH neurons, this agonist would effectively probe the effects of Y1 and Y4 activation in these cells.

Unexpectedly, loose-cell recordings showed that [Leu³¹,Pro³⁴]-NPY could inhibit or excite GnRH neuron firing rate. Of six GnRH neurons exposed to 300 nM [Leu³¹,Pro³⁴]-NPY, two were inhibited (21%) (Fig. 5A), three activated (1.5- to 2.6-fold increase) (Fig. 5, B and C), and the last cell was unresponsive ($N = 5$). At a lower concentration of 100 nM, [Leu³¹,Pro³⁴]-NPY (37) was only found to exert stimulatory effects on three of eight GnRH neurons ($N = 4$) increasing their firing rate between 1.8- and 4.4-fold, whereas the other neurons were unresponsive (Table 1, lower panel).

Experiments using whole-cell and perforated-patch modes found that 300 nM [Leu³¹,Pro³⁴]-NPY evoked membrane depolarization (4.7 ± 1.4 mV) in four of six GnRH neurons with or without associated changes in firing rate (Fig. 5, D and E). The membrane depolarization was maintained (3.4 ± 0.9 mV) in the presence of TTX and amino acid receptor blockers (Fig. 5F) with two of four GnRH neurons responding.

Overall, this set of studies showed that nine of 20 (45%) GnRH neurons were stimulated, and three of 20 (15%) inhibited by [Leu³¹,Pro³⁴]-NPY.

rPP, a selective Y4 agonist, excites GnRH neurons

Because GnRH neurons were stimulated by a Y1/Y4/Y5 agonist but not by a Y5 agonist or a Y1/Y2/Y5 agonist, it seemed likely that the Y4 receptor was responsible for exciting GnRH neurons. We examined this by

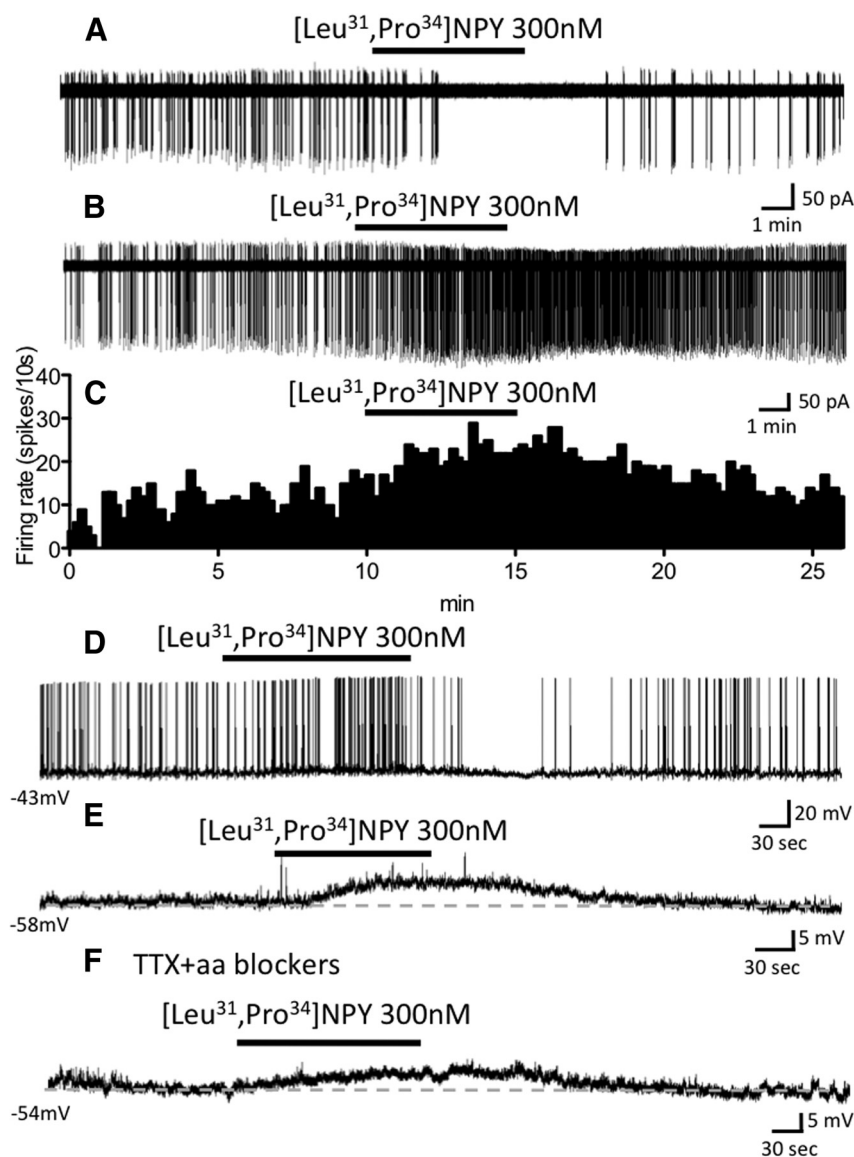


FIG. 5. Y1/Y4/Y5 receptor agonist [Leu³¹,Pro³⁴]-NPY either inhibits or excites GnRH neurons. Loose-patch recordings show either inhibition (A) or excitation (B and C) of GnRH neurons in response to [Leu³¹,Pro³⁴]-NPY. C, Frequency-time histogram illustrative of firing rate displayed by the cell presented above. D, Trace from a perforated-patch recording shows one cell that increased firing transiently, accompanied by depolarization. E, Depolarization induced by [Leu³¹,Pro³⁴]-NPY did not evoke action potentials in some cells. F, The stimulatory effect of [Leu³¹,Pro³⁴]-NPY on membrane potential persists in the presence of TTX and amino acid (aa) blockers.

testing the effects of rPP, a highly selective rat Y4 agonist (38), on GnRH neurons. Loose-patch recordings showed that 30 nM rPP (39) increased the firing rate of three of 11 GnRH neurons ($N = 3$) (Fig. 6A). The magnitude of the response was from 1.6- to 6-fold. Treatment with 300 nM rPP evoked the same response from three of 10 GnRH neurons, although it was not possible to determine the fold increase as all three responsive cells were silent before testing ($N = 5$) (Table 1, lower panel). In all cases, the activation was slow in onset, usually only seen in the immediate washout period (Fig. 6, A and B) and less striking

than that observed with [Leu³¹,Pro³⁴]-NPY. This is probably due to the use of rPP, which differs from mouse PP by two amino acids, and will substantially reduce its potency at the mouse Y4 receptor (38). In perforated-patch experiments, 30 nM rPP significantly depolarized three of six GnRH neurons (3.4 ± 0.9 mV) with accompanied increases in firing rate (Fig. 6, B and C). The addition of TTX and amino acid blockers did not prevent this depolarization in a further two of four GnRH neurons (2.2 ± 0.6 mV) (Fig. 6D).

AgRP exerts variable effects on a small number of GnRH neurons

In rodents, arcuate NPY neurons coexpress AgRP (40), an endogenous MC3R and MC4R (MC3/4R) antagonist (41, 42). Thirteen GnRH neurons ($n = 6$) were tested with 10 nM AgRP (29), with one cell exhibiting a reduction of 38% in firing and three being excited between 1.3- and 10-fold (Table 1, lower panel). At 100 nM, AgRP had very similar actions, with one of 10 GnRH neurons ($N = 4$) being inhibited by 49% (Fig. 7, A and B) and three (Fig. 7C) stimulated between 2.1- and 3.6-fold.

Discussion

Signals of energy sufficiency are necessary for the onset of puberty and maintenance of reproductive function. However, the pathways conveying metabolic information to the neuronal network controlling fertility are only just beginning to be understood. We demonstrate here that approximately 70% of GnRH neurons are excited by α -MSH acting through both MCR3 and MCR4 receptors on GnRH neurons. In contrast, AgRP, the endogenous antagonist at MCR3/4 receptors released by NPY neurons, exhibited variable effects upon GnRH neurons. Other POMC neuron neuropeptides, CART and β -endorphin, had only modest effects on a small number of GnRH neurons. Although the majority of GnRH neurons were modulated by NPY, this was found to involve NPY receptor-dependent excitatory (Y4) and inhibitory

testing the effects of rPP, a highly selective rat Y4 agonist (38), on GnRH neurons. Loose-patch recordings showed that 30 nM rPP (39) increased the firing rate of three of 11 GnRH neurons ($N = 3$) (Fig. 6A). The magnitude of the response was from 1.6- to 6-fold. Treatment with 300 nM rPP evoked the same response from three of 10 GnRH neurons, although it was not possible to determine the fold increase as all three responsive cells were silent before testing ($N = 5$) (Table 1, lower panel). In all cases, the activation was slow in onset, usually only seen in the immediate washout period (Fig. 6, A and B) and less striking

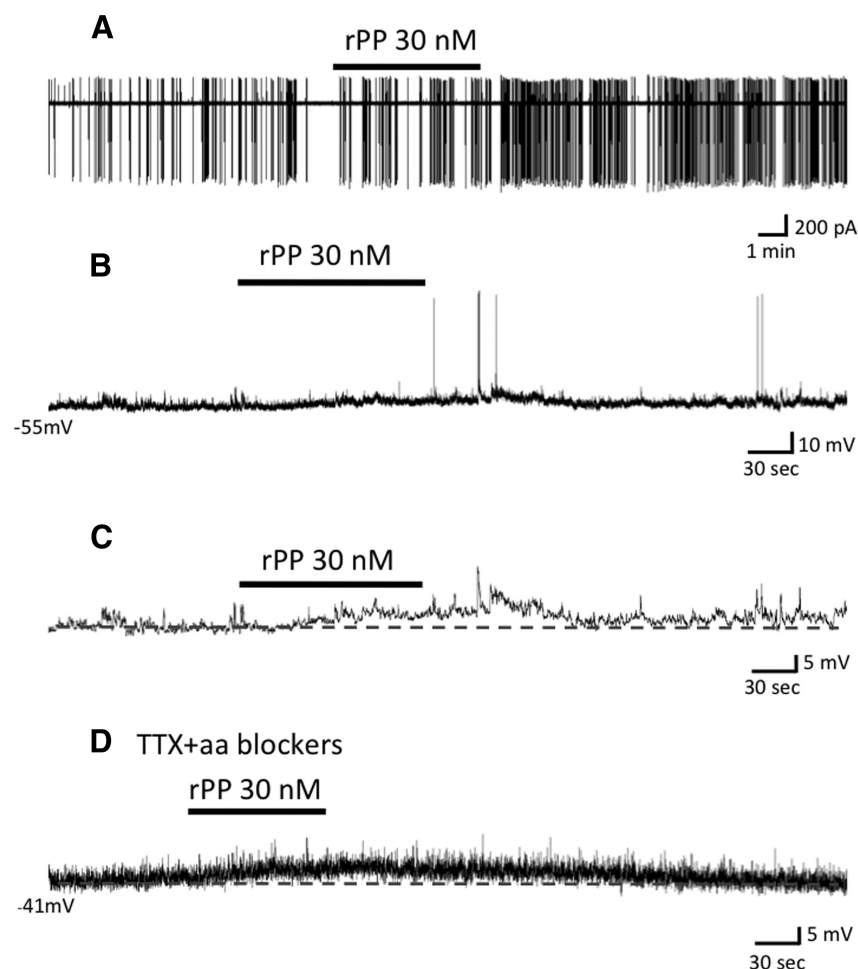


FIG. 6. Y4 receptor agonist rPP excites GnRH neurons. A, Representative recording, in loose-patch configuration, of the late onset activation produced by rPP on GnRH neurons. B and C, Current-clamp recording in perforated-patch mode shows GnRH activation in response to rPP agonist that is accompanied by an increase in membrane potential. C, Membrane potential from the cell above after removal of action potentials by Gaussian low-pass filtering (-3 -dB cutoff at 0.8 Hz). D, Depolarization produced by rPP is preserved in the presence of TTX and amino acid (aa) blockers.

(Y1) actions. As is typical for GnRH neurons, we have found substantial heterogeneity in the response of GnRH neurons to the different POMC and NPY neuron-related peptides. The reasons for this remain unclear but may well result from the existence of intermingled subpopulations of GnRH neurons that have specific functions (43).

Regulation of GnRH neuron excitability by POMC-related neuropeptides

Arcuate POMC neurons use multiple different neuropeptides, including α -MSH, β -endorphin, and CART, to control food intake and energy homeostasis (44, 45). Immunocytochemical studies in rodents indicated that POMC neurons were also likely to be involved in regulating reproduction. POMC neurons project to the general vicinity of GnRH neuron cell bodies (46) and terminals immunoreactive for POMC-related peptides synapse on GnRH neuron cell bodies (18, 47). We now provide evi-

dence for a direct functional POMC neuron input to GnRH neurons in female mice. Although the majority of GnRH neurons were excited by potent, direct actions of α -MSH, only relatively weak and inconsistent actions of the other POMC neuropeptides were found on GnRH neurons. Approximately 25% of GnRH neurons responded to CART with a modest increase in firing rate, but this was only evident at the highest concentration of CART tested ($1 \mu\text{M}$). Fewer (18%) GnRH neurons responded to 500 nM β -endorphin with a suppression in firing. Because a previous study in the guinea pig had reported that 100% of postidentified GnRH neurons were inhibited by the μ -opiate receptor agonist DAMGO (48), we tested GnRH neurons with 1 or $10 \mu\text{M}$ DAMGO but found this peptide to have no effects. The relative inability of β -endorphin to influence mouse GnRH neuron firing found here is in good agreement with studies that have found no evidence for μ -opiate receptors to be expressed by GnRH neurons (49, 50). Together, these studies show that α -MSH is very likely to be the most important neuropeptide used by POMC neurons to regulate GnRH neuron excitability. The coreleased peptides β -endorphin and CART would appear to have little effect, although we cannot rule out pre-

synaptic actions on POMC terminals synapsing on GnRH neurons.

We demonstrate here that α -MSH acts through both MC4R and MC3R receptors. The MC4R agonist produced a long-lasting depolarization in 60–70% of GnRH neurons, and this effect persisted in presence of TTX and amino acid ionotropic receptor antagonists, indicating the direct postsynaptic activation of GnRH neurons. A recent study has found that approximately 50% of GnRH neurons in male and female mice express MCR4 transcripts (51). The MC3R agonist was found to have the same stimulatory effect, although in this case, the membrane depolarization was not maintained in whole-cell mode and required perforated patch recordings for analysis. This suggests that the intracellular pathway coupling MC3R receptors (52, 53) to membrane depolarization is more sensitive than that of MCR4 receptors (54, 55) to intra-

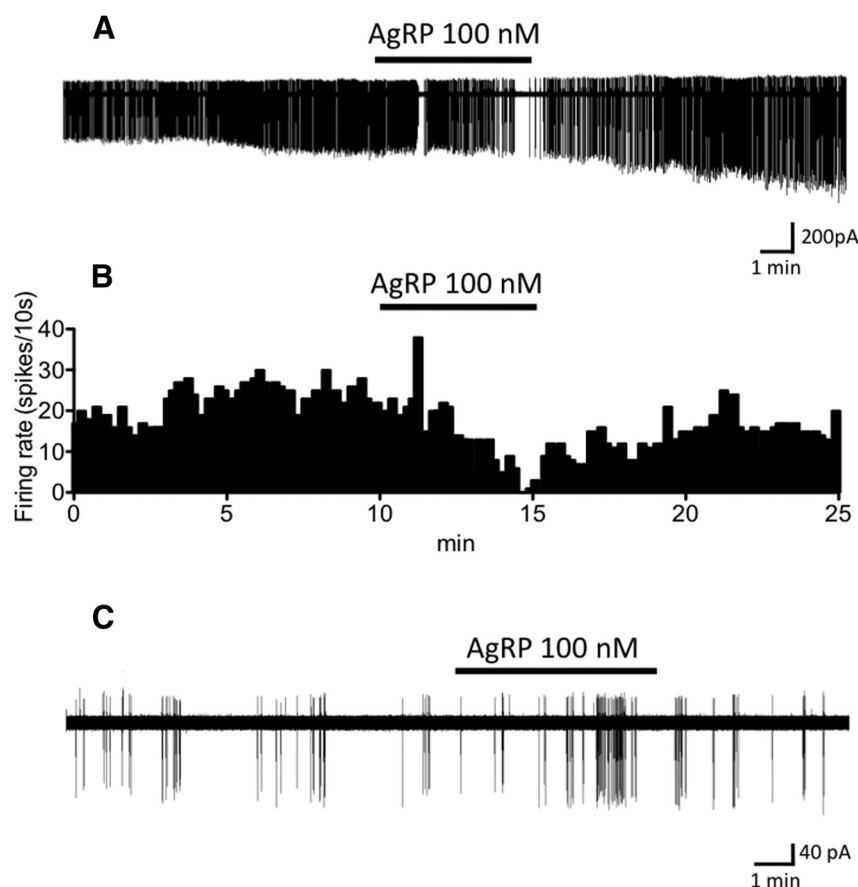


FIG. 7. AgRP either inhibits or excites GnRH neurons. AgRP either decreases (A and B) or increases (C) cell firing in a subpopulation of GnRH neurons. Representative recordings, in loose-patch mode, showing a significant inhibitory (A) or stimulatory (C) effect produced by AgRP on GnRH neurons. B, Frequency-time histogram illustrative of firing rate displayed by the cell shown in A.

cellular dialysis of the GnRH neuron with the patch pipette contents (56). Precisely how this comes about is not known, although the MCR3 and MCR4 receptors do use different second messenger pathways; for example only MCR3 uses inositol phosphate (55).

Although an important role exists for MCR3 and MCR4 receptors in controlling metabolism (57, 58), their function in the control of reproduction is less clear. Mice with targeted mutations of MCR3 or MCR4 are fertile but produce smaller, infrequent litters (58, 59). It is possible that this relatively mild reproductive phenotype results from compensation of one MCR for the other in knockout mouse lines. This proposal is compatible with the demonstration here that GnRH neurons express both MCR3 and MCR4 receptors and that they have very similar actions on GnRH neuron excitability.

Because MCR3/MCR4 activation depolarizes GnRH neurons, we anticipated that AgRP, the endogenous MCR3/4 antagonist (60), would have either no effect or an inhibitory action on GnRH neuron firing depending upon the level of on-going endogenous α -MSH signaling in the

brain slice preparation. Although we found a small number of GnRH neurons ($\sim 10\%$) inhibited by AgRP, suggesting a low degree of tonic α -MSH activity at the GnRH neuron, we were surprised to observe that approximately 25% of GnRH neurons were excited by this neuropeptide. Although this excitation may result from indirect effects, AgRP is known to exert effects on neural activity independent of MCR3/4 receptors in the brain (29, 61, 62). Interestingly, mice in which both MCR3 and MCR4 are antagonized by overexpression of AgRP are infertile (63), and a very recent study has demonstrated that MCR3/4 activation of peripubertal GnRH neuron firing is abolished by AgRP (51).

Regulation of GnRH neuron excitability by NPY

Previous studies have reported that GnRH neurons are likely to receive direct inputs from NPY neurons and that these afferents originate from the brain stem and ARN (17, 46, 64). We report here that NPY is able to exert both stimulatory and inhibitory actions on adult female GnRH neurons through Y4 and Y1 receptors, respectively. The complexity of NPY receptor pharmacology

required us to define the receptors involved in these responses by subtractive analysis. Initially, we found that pNPY, active at Y1/Y2/Y5 receptors, robustly suppressed the firing of approximately 50% of GnRH neurons without a change in resting membrane potential. Because a Y5 receptor antagonist was found to have no effect, we supposed that the inhibitory response was mediated by Y1 or Y2 receptors. To differentiate this further, we used the [Leu³¹,Pro³⁴]-NPY agonist active at Y1, Y4, and Y5 receptors. To our surprise, [Leu³¹,Pro³⁴]-NPY stimulated approximately 50% and inhibited a further 30% of GnRH neurons. This unexpected stimulatory response resulted from the direct activation of postsynaptic receptors on GnRH neurons. Because it seemed most likely that Y4 receptors were responsible for exciting GnRH neurons, we tested rPP, selective for the Y4 receptor (65) and found it to evoke direct depolarizing actions in approximately 50% of GnRH neurons. Together, these studies show that NPY inhibits GnRH neuron excitability through Y1 receptors and stimulates them through Y4 receptors.

Previous studies have shown that NPY modulates γ -aminobutyric acid-ergic inputs to mouse GnRH neurons as well as calcium transients in cultured embryonic GnRH neurons through the Y1 receptor (66, 67). Also, in rats, NPY hyperpolarizes GnRH neurons by NPY through the Y5 receptor (19). Because we were unable to detect any effects of Y5 activation in the mouse, the latter observation suggests substantial species variation in the NPY regulation of GnRH neurons. Although there is evidence for stimulatory effects of Y4 receptor activation on the gonadotrophic axis (68, 69), the deletion of Y4 signaling partially rescues infertility in ob/ob mice, suggesting a possible suppressive effect for Y4 signaling (70). However, this improvement in reproductive function could be attributed to the normalization of the corticosterone levels that has been recently revealed as main cause for the infertility in ob/ob mice (71). On the other hand, besides the direct stimulatory effects of Y4 signaling on GnRH neurons demonstrated here, other central and peripherals actions influencing the reproductive axis cannot be excluded. For example, recent analyses suggest that fasting indirectly inhibits the gonadotrophic axis through Y4 receptors (72).

The observation that GnRH neurons are likely to express Y4 receptors is intriguing. PP is considered the endogenous ligand for the Y4 receptor, and although peripheral PP administration can activate Y4 receptors, PP is not synthesized in the brain (73). Furthermore, NPY affinity for the Y4 receptor is over 1000-fold lower than that for Y1 receptors (74). This suggests that the effects of endogenous NPY on GnRH neurons would be dominated by inhibitory Y1 receptor-mediated events. However, one possible role for Y4 receptors on GnRH neurons may be through sensing circulating PP concentrations in the periphery, because these neurons extend their dendrites outside the blood-brain barrier (75).

Integration of metabolic and reproductive neuronal circuitries

As might be expected, multiple pathways exist for the metabolic regulation of the neuronal network controlling reproduction. In addition to direct effects of glucose concentration on GnRH neuron activity (76), inputs from hypothalamic melanin-concentrating hormone neurons (77) and leptin-sensing ventral premammillary nucleus neurons (9–11) play a role. We now provide functional evidence that arcuate POMC and NPY neurons represent another such input. The POMC and NPY neurons are in an ideal position to integrate a wide variety of metabolic parameters, including leptin, insulin, ghrelin, and leucine concentrations (78–80), and to coordinate feeding and reproduction. Conditions favoring high NPY and low POMC neuron activity, as found at times of poor nutrition

(81), will stimulate feeding and, as shown here, provide the necessary suppression of fertility by decreasing GnRH neuron excitability through reduced stimulatory α -MSH drive and increased inhibitory Y1 receptor-mediated NPY release. The relative contribution of the POMC/NPY inputs to GnRH neurons in mediating metabolic cues has yet to be established, although it is notable that the infertility of leptin receptor deleted mice can be rescued by breeding them onto either NPY or Y1 receptor knockout mouse lines (82) or by ablating AgRP (51). Because NPY- and MSH-related compounds are presently being developed as antiobesity therapies (65), the present results suggest that it will also be important to consider their effects on human fertility.

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