Kisspeptin Directly Regulates Neuropeptide Y Synthesis and Secretion via the ERK1/2 and p38 Mitogen-Activated Protein Kinase Signaling Pathways in NPY-Secreting Hypothalamic Neurons

Ginah L. Kim, Sandeep S. Dhillon, and Denise D. Belsham

Departments of Physiology (G.L.K., S.S.D., D.D.B.), Obstetrics and Gynaecology (D.D.B.), and Medicine (D.D.B.), University of Toronto, and Division of Cellular and Molecular Biology (D.D.B.), Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario, Canada M5S 1A8

Kisspeptin is a key component of reproduction that directly stimulates GnRH neurons. However, recent studies indicate that kisspeptin can indirectly stimulate GnRH neurons through unidentified afferent networks. Neuropeptide Y (NPY) is another key reproductive hormone that is an afferent stimulator of GnRH neurons. Herein, we report kisspeptin receptor Kiss1r mRNA expression in native NPY neurons FAC-sorted from NPY-GFP transgenic mice. Thus, we hypothesized that kisspeptin indirectly stimulates GnRH neurons through direct regulation of NPY neurons. Using hypothalamic NPY-secreting cell lines, we determined that kisspeptin stimulates NPY mRNA expression and secretion in the mHypoE-38 cells, but not the mHypoE-42 cells, using quantitative RT-PCR and enzyme immunoassays. Furthermore, agouti-related peptide, ghrelin, neurotensin, or Kiss1r mRNA expression was not changed upon exposure to kisspeptin in either cell line. These results concur with our previous work identifying the mHypoE-38 cell line as a putative reproductive NPY neuron and the mHypoE-42 cell line as a potential feeding-related NPY neuron. In the mHypoE-38 cells, kisspeptin activated the ERK1/2 and p38 MAPK kinases as shown by Western blot analysis. Moreover, inhibiting the ERK1/2 and p38 pathways with U0126 and SB239063, respectively, prevented kisspeptin induction of NPY mRNA expression and secretion. Altogether, we find that kisspeptin directly regulates NPY synthesis and secretion via the ERK1/2 and p38 MAPK pathways in a NPY-secreting cell line, and we propose NPY neurons as an afferent network by which kisspeptin indirectly stimulates GnRH secretion. **(*Endocrinology* 151: 5038–5047, 2010)**

Kisspeptin is now widely acknowledged as a key neuropeptide hormone involved in initiating puberty and maintaining reproductive function. Originally discovered to suppress metastasis, the role of kisspeptin in reproduction was first identified with the finding that mutations in the kisspeptin receptor, Kiss1r, were associated with idiopathic hypogonadotropic hypogonadism in human patients (1, 2). *Kiss1r* and *Kiss1* knockout mice were found to have the same reproductively impaired phenotype, with delayed pubertal development, infertility, and reduced sexual behavior (2–4). A series of studies into the precise mechanisms of kisspeptin action subsequently revealed that kisspeptin increases gonadotropin secretion through direct stimulation of GnRH neurons. Specifically, studies have demonstrated that kisspeptin neurons project to GnRH neurons in the preoptic area and median eminence (5, 6), and approximately 70% of GnRH neurons express Kiss1r (7). Kisspeptin has also been found to induce GnRH, LH, and FSH release (8–12); increase GnRH neuronal firing (13, 14); and stimulate GnRH mRNA expression and secretion in GnRH-secreting neuronal cell lines (15). Collectively, these studies provide strong evidence that kisspeptin exerts its control of the reproductive axis through the direct regulation of GnRH neurons.

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-0521 Received May 10, 2010. Accepted July 19, 2010.
First Published Online August 4, 2010

Abbreviations: AgRP, Agouti-related peptide; DMSO, dimethylsulfoxide; EIA, enzyme immunoassay; ER, estrogen receptor; FAC, fluorescence-activated cell; FACS, FAC sorting; Gβ, G protein β; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; Kiss1r, kisspeptin receptor; MEK, MAPK kinase; NPY, neuropeptide Y; NSE, neuron-specific enolase; NT, neurotensin; PI3K, phosphatidylinositol-3-kinase; POMC, proopiomelanocortin; TBS-T, Tris-buffered saline with Tween.

To date, studies have largely focused on the direct regulation of kisspeptin on GnRH neurons. Although this mechanism has been unequivocally demonstrated (5–15), the possibility that kisspeptin indirectly stimulates GnRH neurons through afferent neuronal networks has not been excluded. In fact, Kiss1r is expressed in other regions of the hypothalamus not associated with GnRH (7, 16). One study found that blockade of fast synaptic transmission significantly reduced, but did not completely abrogate, the response to kisspeptin in GnRH neurons. In addition, kisspeptin was found to increase the firing rate of non-GnRH neurons in the medial preoptic area (17). These findings indicate that the effects of kisspeptin on GnRH are both direct and indirect, and therefore, kisspeptin may regulate GnRH indirectly through an as yet unidentified afferent network.

Neuropeptide Y (NPY) is another key hormone that is well established as a major regulator of the reproductive axis; therefore, we considered the possibility that kisspeptin indirectly regulates GnRH through direct activation of NPY neurons. NPY neurons in the arcuate nucleus of the hypothalamus have been found to project to GnRH neurons (18, 19), and morphological evidence indicates colocalization of NPY receptors and GnRH neurons (18). Both *in vivo* and *in vitro* studies have demonstrated that NPY stimulates GnRH secretion (20–26), because NPY infusion into the third ventricle of ewes significantly elevated GnRH levels in the median eminence (23), and exposure of NPY to GT1-7 cells significantly increases GnRH secretion (24). Although the stimulatory effect of NPY on GnRH neurons has been well-documented, NPY can also have an inhibitory action on GnRH release, which is associated with changes in steroidal environment, stage of development, and species (27–32). For instance, intrahypothalamic perfusion of NPY decreased mean GnRH levels in ovariectomized rats, whereas the same NPY perfusion stimulated mean GnRH levels in intact rabbits (32). Altogether, NPY is a potent regulator of the reproductive axis, with both positive and negative regulation demonstrated depending on the experimental conditions.

Our goal was to determine whether kisspeptin can directly regulate NPY synthesis and secretion, potentially resulting in GnRH regulation. It is not yet known whether NPY neurons *in situ* express the kisspeptin receptor Kiss1r, and thus we used fluorescence-activated cell (FAC)-sorted NPY-green fluorescent protein (GFP) neurons from transgenic mice to explore this possibility. Due to the inherent cellular heterogeneity of the hypothalamus, we employed clonal, hypothalamic NPY-secreting cell lines, mHypoE-38 and mHypoE-42, to further study the potential mechanisms involved in this process. These clonal, murine cell lines were immortalized via the retroviral transfer of the simian virus 40 T-antigen (33). Both lines have been previously characterized (33, 34), and the secretory and expression profiles were further evaluated herein. We found that kisspeptin has direct effects on NPY mRNA expression and secretion in the hypothalamic mHypoE-38 cell line, but not the mHypoE-42 cell line. We further investigated the intracellular signaling pathways activated by kisspeptin. Our findings represent the first line of evidence that kisspeptin directly regulates NPY synthesis and secretion, providing a putative mechanism whereby kisspeptin indirectly activates GnRH neurons.

Materials and Methods

Cell culture and reagents

mHypoE-38 and -42 cells were cultured in a monolayer in DMEM (Invitrogen Life Technologies, Burlington, Ontario, Canada), supplemented with 5% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT) and 1% penicillin/streptomycin (Invitrogen Life Technologies) and maintained at 37 C in 5% CO_2, as previously described (33). The mHypoE-42 cell line was generated using the same technique to generate the mHypoE-38 cell line, but subcloned after the initial report of the cell lines (33). Phenotypic profiles of these lines can be found on the website http://cellutionsbiosystems.com or from previous manuscripts (33, 34). Before peptide treatments, cell culture media was replaced with 1% fetal bovine serum and 1% penicillin/streptomycin for a minimum of 3 h. Kiss-10 peptide was purchased from Phoenix Pharmaceuticals (Burlingame, CA) and dissolved in water (10 nM final concentration). The MAPK kinase (MEK)1/2 inhibitor U0126 and the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 were obtained from Cell Signaling Technology Inc. (Danvers, MA). The p38 inhibitor SB239063 was obtained from Tocris Bioscience (Ellisville, MO). All inhibitors were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Oakville, Ontario, Canada) at a final concentration of 25 μM (U0126 and LY294002) or 10 μM (SB239063), and applied to neurons 1 h before kiss-10 treatment. The G protein β (Gβ) and phopho-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the phospho-ERK1/2 and phospho-Akt antibodies were purchased from Cell Signaling Technology Inc.

FAC sorting (FACS)

The NPY-GFP mice [strain B6.FVB-Tg(Npy-hrGFP)1Lowl/J], generated by Dr. Bradford Lowell (Beth Israel Deaconess Medical Center, Boston, MA) and available through The Jackson Laboratory (Bar Harbor, ME), were housed under standard viviarium conditions in a 12-h light, 12-h dark cycle with food and water available *ad libitum*. All procedures were conducted in accordance with the regulations of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee. NPY-GFP transgenic mouse hypothalami from 10- to 20-wk-old mice were dissected individually and stored in Hank's balanced salt solution supplemented with 0.5 mM EDTA and 1% BSA. Cells were dispersed by trituration through a 21-gauge needle and passed through 40-μM filter tubes. Cells were sorted on a BD FACSAria cell sorter (Becton Dickinson, Franklin Lakes, NJ) with a 100-μm nozzle tip and sheath pressure at 20 ψ with a purity greater than 95%. NPY-GFP cells were sorted on GFP fluorescence after gating to remove

cell aggregates. All FACS was performed in the Faculty of Medicine Flow Cytometry Facility, University of Toronto.

Reverse transcription PCR (RT-PCR)

Each cell line was analyzed for the expression of specific markers by RT-PCR. Total RNA was isolated from mHypoE-38 and -42 cells and mouse hypothalamus using the guanidinium thiocyanate phenol chloroform extraction method and assessed for purity and concentration using spectrophotometric analysis (Ultraspec3000; Amersham Pharmacia Biotech, Piscataway, NJ). RNA samples were amplified using a one-step RT-PCR Kit (QIAGEN, Mississauga, Ontario, Canada) according to the manufacturer's instructions. PCR was conducted according to the following: 95 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min (40 cycles). A total of 200 ng of RNA template from each cell line was used for each reaction. All PCR-amplified products were visualized on 2% agarose gels containing ethidium bromide (final concentration, 0.05 mg/ml) under UV light. A 50-bp DNA Ladder (Fermentas Life Science, Burlington, Ontario, Canada) was used to determine product size. Refer to Ref. 33 for neuron-specific enolase (NSE), T-antigen, glial fibrillary acidic protein (GFAP), NPY, agouti-related peptide (AgRP), proopiomelanocortin (POMC), neurotensin (NT), and ghrelin primer sequences and Ref. 34 for estrogen receptor (ER)α and ERβ primer sequences. Kiss1r primer sequences are as follows: sense, 5′-tgg ttc ccc tgt ttt tcg cta-3′; antisense, 5′-cag cgg gaa cac agt cac ata-3′.

Quantitative RT-PCR

Total cellular RNA was isolated from the mHypoE-38 and -42 cells by the guanidinium isothiocyanate phenol chloroform extraction method. cDNA was made using the Applied Biosystems (Foster City, CA) High Capacity cDNA Reverse Transcriptase Kit. Analysis of NPY, POMC, AgRP, Kiss1r, NT, and ghrelin mRNA expression was completed using real-time RT-PCR. Real-time RT-PCR was performed using a SYBR green mix containing 0.3× Sybr green dye, 1× ROX (a passive reference dye), 1× buffer, 3 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, and 0.5 U Platinum Taq (all from Invitrogen Life Technologies) with approximately 100 ng template and run on the Applied Biosystems Prism 7000 real-time PCR machine. NPY primer sequences are as follows: SYBR sense, 5′-aat cag tgt ctc agg gct gga t-3′; SYBR antisense, 5′-ccg ctc tgc gac act aca tc-3′. POMC primer sequences are as follows: SYBR sense, 5′-aag agc agt gac taa gag agg cca-3′; SYBR antisense, 5′-aca tct atg gag gtc tga agc agg-3′. AgRP primer sequences are as follows: SYBR sense, 5′-cgg agg tgc tag atc cac aga; SYBR antisense, 5′-agg act cgt gca gcc tta cac-3′. Kiss1r sequences are as follows: SYBR sense, 5′-cac atg cag aca gtt acc aac ttc t-3′; SYBR antisense, 5′-cac gca gca cag tag gaa agt g-3′. Preproghrelin sequences are as follows: SYBR sense, 5′-gga gga gct gga gat cag gtt; SYBR antisense, 5′-ggc ccg gcc atg ctg ct-3′. Histone sequences are as follows: SYBR sense, 5′-cgc ttc cag agt gca gct att-3′; SYBR antisense, 5′-atc ttc aaa aag gcc aac cag at-3′. Gene-specific TaqMan primers and probe for NT were purchased from Applied Biosystems. Real-time RT-PCR values were normalized to histone at the corresponding time points.

Western blot analysis

mHypoE-38 and -42 cells were grown to 90% confluence, serum-starved for 4 h, and then treated with kisspeptin (10 nM) or vehicle. The cells were washed with ice-cold PBS and harvested at 5, 15, 30, and 60 min using a 1× lysis buffer (Cell Signaling Technology Inc.) supplemented with 1 mM phenylmethylsulfonylfluoride and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). Cell lysates were centrifuged at 14,000 rpm for 10 min at 4 C, and the supernatant was stored at −80 C. Concentration of the protein was measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL). A total of 30 μg of protein was resolved on an 8% sodium dodecyl sulfate-polyacrylamide gel and blotted overnight onto immunoblot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The blots were blocked with 5% BSA (Sigma-Aldrich) in Tris-buffered saline with 0.1% Tween (TBS-T) for 30–60 min, washed three times with TBS-T, and incubated overnight at 4 C with primary antibodies phospho-ERK1/2 (1:1000), phospho-p38 (1:1000), phospho-Akt (1:1000), and Gβ (1:5000). Blots were then washed three times with TBS-T and incubated with horseradish peroxidase-labeled secondary goat antirabbit antisera (1:5000; Cell Signaling Technology Inc.) or sheep antimouse (1:5000; Cell Signaling Technology Inc.) for 1–2 h at room temperature. Membranes were visualized with enhanced chemiluminescence (ECL kit; GE Healthcare, Chalfont St. Giles, UK).

Enzyme immunoassay (EIA)

mHypoE-38 and -42 cells were grown to 90% confluence, and cell culture medium was replaced with serum-free DMEM containing 1% penicillin/streptomycin for a minimum of 4 h before treatment with 10 nM kisspeptin, 60 mM KCl, or vehicle alone. Media was collected after 15 min (KCl) or 1 h (kisspeptin), and the concentration of NPY was analyzed using an NPY-specific EIA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA) according to the manufacturer's protocol.

Statistical analysis

Data are presented as the mean ± SEM and analyzed using SigmaStat software (Systat Software, San Jose, CA). Statistical significance was determined using a Student's *t* test (secretion studies, KCl and kisspeptin treatment), one-way ANOVA followed by a *post hoc* Student Newman-Keuls test (inhibitor studies), or two-way ANOVA followed by a *post hoc* Bonferroni test (mRNA expression studies, kisspeptin treatment; protein phosphorylation studies). Experiments were performed on three to eight separate occasions. Data were considered statistically significant when $P < 0.05$.

Results

Expression of Kiss1r mRNA in flow cytometry-isolated NPY-GFP neurons

Kiss1r is expressed not only in the GnRH neuron, but also in other regions of the hypothalamus (7, 16). The function of Kiss1r in these regions is currently unknown. We isolated native NPY-GFP neurons from the hypothalamus of the transgenic mouse using FACS and found expression of Kiss1r mRNA as shown by RT-PCR (Fig. 1). Total RNA from mouse hypothalamus was used as a positive control, and reactions containing no RNA template were used as a negative control for one-step RT-PCRs. These results indicate that native NPY neurons in the mouse hypothalamus

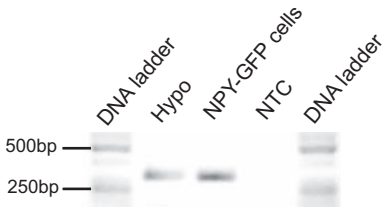


FIG. 1. Expression of Kiss1r mRNA in NPY-GFP neurons isolated from the hypothalamus of the transgenic mouse using FACS. RNA harvested from NPY-GFP neurons was used as a template for one-step RT-PCR with primers specifically designed to amplify Kiss1r. RNA from mouse hypothalamus (Hypo) was used as a positive control, and a reaction containing no RNA template (NTC) was used as a negative control for one-step RT-PCRs. Amplicon size: Kiss1r, 314 bp.

express the kisspeptin receptor Kiss1r and may therefore respond to kisspeptin in a physiological setting.

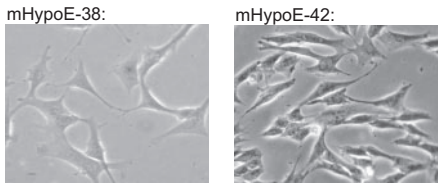
Expression of Kiss1r mRNA and hypothalamic markers in NPY-secreting hypothalamic cell lines

Studying the direct regulation of specific neurons in the hypothalamus is difficult due to the inherent cellular heterogeneity and complexity of neuronal circuits within. Thus, to optimally study the direct regulation of NPY neurons by kisspeptin, we employed clonal, immortalized, hypothalamic cell lines, mHypoE-38 and -42, derived from the embryonic mouse. The generation (33) and characterization (33, 34) of these cells has been previously reported. Briefly, cells were immortalized through the retroviral transfer of the simian virus 40 T-antigen oncogene and subcloned until a homogeneous cell population was obtained. Each cell line displays neuronal morphology and has a distinct phenotypic profile (Fig. 2A). Using RT-PCR, both lines were confirmed for the presence of NSE and T-antigen and the absence of GFAP. Further screening for hypothalamic neuropeptides and receptors determined that both cell lines express mRNA for NPY, AgRP, ghrelin, ERα, and ERβ, but not POMC. NT mRNA expression was found in the mHypoE-42 but not the mHypoE-38 cell line. Importantly, both cell lines express Kiss1r mRNA, predicting that these lines are responsive to kisspeptin and are therefore suitable models to investigate the direct regulation of NPY mRNA expression and secretion by kisspeptin (Fig. 2B). These cell lines also secrete NPY basally and in response to KCl-induced depolarization, as shown by an NPY-specific EIA (Fig. 2C).

Kisspeptin regulates NPY mRNA expression and secretion in the mHypoE-38 cell line

Currently, functions of Kiss1r-expressing non-GnRH neurons in the hypothalamus remain unclear. We have located Kiss1r mRNA expression in FAC-sorted native NPY-GFP cells and two NPY-secreting hypothalamic cell lines. Thus, we tested the hypothesis that kisspeptin directly regulates NPY synthesis and secretion using the

A



B

Marker	mHypoE-38	mHypoE-42
NSE	+	+
T-Ag	+	+
GFAP	-	-
NPY	+	+
AgRP	+	+
POMC	-	-
NT	-	+
Ghrelin	+	+
GPR54	+	+
ERα	+	+
ERβ	+	+

C

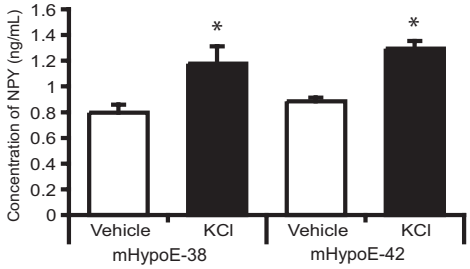


FIG. 2. A, Representative phase-contrast micrographs of clonal, immortalized, murine, hypothalamic cell lines mHypoE-38 and mHypoE-42. Each cell line displays neuronal morphology and has a distinct phenotypic profile. B, Expression of neuronal markers, neuropeptides, and receptors was analyzed in both cell lines using RT-PCR. Positive controls using hypothalamus RNA and negative controls containing no RNA template were performed and validated for each gene. Listed in the table is the presence (+) or absence (−) of specific genes. T-Ag, T-antigen. C, mHypoE-38 and mHypoE-42 cells were treated with 60 mM KCl or water. Media was collected at 15 min after treatment, and the concentration of NPY was analyzed using a NPY-specific EIA. Data are shown as mean ± SEM for three to six independent experiments. *, $P = 0.003$.

NPY-secreting mHypoE-38 and -42 cell lines. Cells were treated with the kiss-10 peptide (10 nM) and harvested after 4, 8, and 24 h. Using quantitative RT-PCR, we found that NPY mRNA expression was significantly increased by kiss-10 treatment at the 24-h timepoint in the mHypoE-38 cells (vehicle, 0.74 ± 0.09, *vs.* kisspeptin, 1.29 ± 0.12). NPY mRNA expression was not significantly regulated by kiss-10 at any timepoint in the mHypoE-42 cells (Fig. 3A). Subsequently, we tested whether kisspeptin could rapidly regulate NPY secretion. We found that kiss-10 treatment (10 nM) for 1 h significantly stimulated NPY secretion in the mHypoE-38 cells (vehicle, 1.00 ± 0.05, *vs.* kisspeptin, 1.26 ± 0.07), but not the mHypoE-42 cells, as determined by an NPY-specific EIA (Fig. 3B). These findings suggest that kisspeptin can act directly on the NPY neuron to rapidly induce NPY secretion and increase NPY mRNA over long-term exposure.

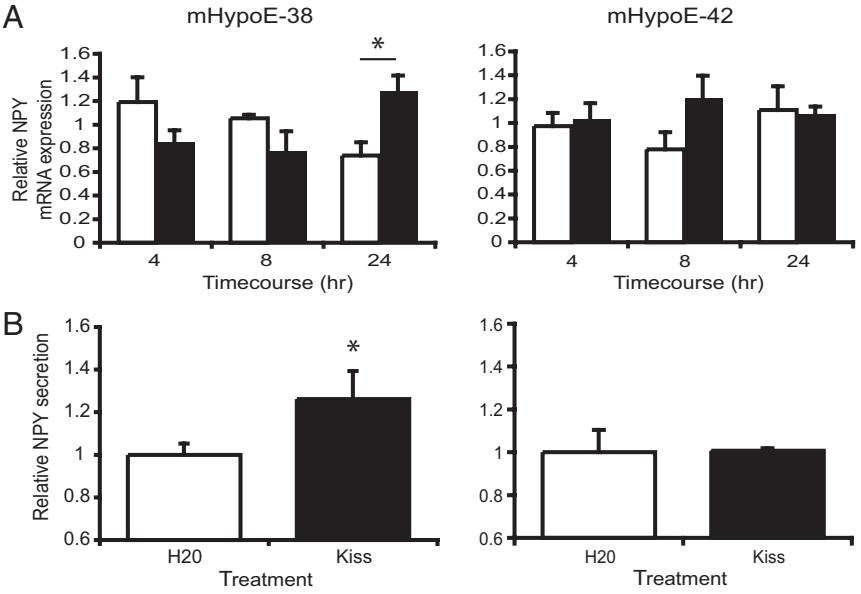


FIG. 3. A, mHypoE-38 and mHypoE-42 cells were treated with kisspeptin (10 nM) over a 24-h time course. NPY mRNA expression was determined using real-time RT-PCR, and levels were normalized to histone H3 (n = three to eight independent experiments). B, mHypoE-38 and mHypoE-42 cells were treated with kisspeptin (10 nM) for 1 h. Concentration of NPY in the media was measured using an EIA. NPY secretion levels were set to 1.0 (n = three to eight independent experiments). Data are shown as mean ± SEM. *, $P < 0.05$. *White bar*, Vehicle control; *black bar*, kisspeptin treatment.

Kisspeptin does not regulate AgRP, ghrelin, NT, or Kiss1r mRNA expression in the mHypoE-38 and mHypoE-42 cells

We investigated whether kisspeptin regulates other important hypothalamic neuropeptides and receptors in the mHypoE-38 and -42 cell lines. Cells were treated with kiss-10 over a 24-h time course, and it was found that AgRP, ghrelin, NT, and Kiss1r mRNA expression were not significantly regulated by kisspeptin in both the mHypoE-38 and -42 cells (Fig. 4). Because treatment with kisspeptin in the mHypoE-42 neurons did not produce a significant effect in any of the genes examined, we sought to confirm the functionality of the kisspeptin receptor in this cell line. We treated the mHypoE-42 cells with 10 nM kiss-10 and harvested protein at the 5, 15, 30, and 60-min timepoints. Using Western blot analysis, we found that ERK1/2 phosphorylation was significantly activated after 5 min (vehicle, 1.00 ± 0.02, *vs.* kisspeptin, 2.04 ± 0.04), indicating that Kiss1r is indeed functional in this cell line (Fig. 5).

Kisspeptin phosphorylates ERK1/2 and p38 in the mHypoE-38 cell line

Next, we aimed to determine the signaling pathways activated by kisspeptin in an endogenous Kiss1r-expressing cell line. The mHypoE-38 neurons were treated with 10 nM kiss-10 over a 60-min time course, and we found that phosphorylation of the ERK1/2 and p38 signaling kinases was significantly activated at the 15- and 60-min timepoints, respectively, as shown by Western blot analysis (ERK1/2, vehicle 1.00 ± 0.12 *vs.* kisspeptin 1.61 ± 0.14; p38, vehicle 1.00 ± 0.05 *vs.* kisspeptin 1.93 ± 0.12). Phosphorylation of Akt was also investigated, but was not significantly altered by kiss-10 treatment (Fig. 6).

The ERK1/2 inhibitor U0126 and p38 inhibitor SB239063 attenuate kisspeptin-mediated induction of NPY mRNA expression and secretion

To determine whether the MEK-ERK and p38 signaling pathways mediate the regulation of NPY by kisspeptin, we used the MEK-specific (U0126) and p38-specific (SB239063) inhibitors. The mHypoE-38 cells were pretreated with the inhibitors for 1 h, followed by cotreatment with inhibitors and 10 nM kiss-10 for 24 h. RNA was then harvested and analyzed using quantitative RT-PCR. We determined that both U0126 (vehicle, 0.63 ± 0.27, *vs.* kisspeptin, 0.59 ± 0.15) and SB239063 (vehicle, 0.74 ± 0.07, *vs.* kisspeptin, 0.78 ± 0.17) significantly attenuated the kisspeptin-mediated induction of NPY mRNA expression (vehicle, 0.74 ± 0.09, *vs.* kisspeptin, 1.29 ± 0.12) (Fig. 7A). Next, we investigated whether the MEK-ERK and p38 pathways are also involved in the kisspeptin-mediated regulation of NPY secretion. Similar to the gene expression studies, the mHypoE-38 cells were pretreated with inhibitors for 1 h, followed by cotreatment with inhibitors and 10 nM kiss-10, and media from the cells was collected 1 h after kiss-10 treatment. Using an NPY-specific EIA, we found that both U0126 (vehicle, 1.01 ± 0.05, *vs.* kisspeptin, 0.98 ± 0.04) and SB239063 (vehicle, 1.17 ± 0.02, *vs.* kisspeptin, 1.17 ± 0.07) prevented the induction of NPY secretion by kisspeptin (vehicle, 1.00 ± 0.05, *vs.* kisspeptin, 1.26 ± 0.07) (Fig. 7B). Altogether, these results indicate that the MEK-ERK and p38 MAPK pathways are essential in the regulation of NPY mRNA expression and secretion by kisspeptin.

Discussion

Kisspeptin is an important reproductive neuropeptide that positively regulates the hypothalamic-pituitary-gonadal axis through activation of GnRH neurons (8–15). In this

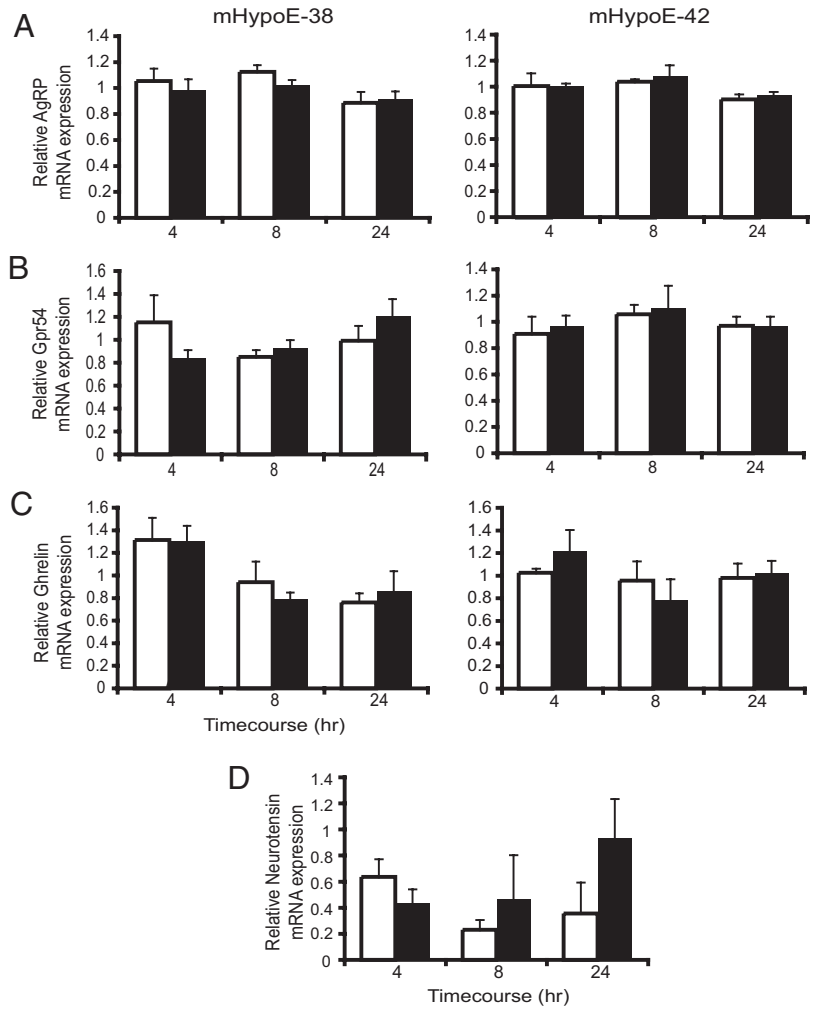


FIG. 4. mHypoE-38 and mHypoE-42 cells were treated with kisspeptin (10 nM) over a 24-h time course. AgRP (A), Kiss1r (B), ghrelin (C), and NT (D) mRNA expression was determined using real-time RT-PCR, and levels were normalized to histone H3 (n = three independent experiments). Data are shown as mean ± SEM. *White bar*, Vehicle control; *black bar*, kisspeptin treatment.

study, we investigated the indirect effect of kisspeptin on the GnRH neuron, as evidenced by Pielecka-Fortuna *et al.* (17). Specifically, we sought to identify the afferent neuronal network that responds to kisspeptin and stimulates GnRH secretion. NPY neurons are found to innervate GnRH cell bodies and terminals (18, 19), and numerous studies have confirmed that NPY stimulates the reproductive axis (21–24). For the first time, we detected Kiss1r mRNA expression in native NPY-GFP neurons FAC-sorted from the hypothalamus of the NPY-GFP transgenic mouse, indicating that hypothalamic NPY neurons may respond to kisspeptin in an *in vivo* setting. Thus, we hypothesized that NPY neurons at least partially form the afferent network through which kisspeptin indirectly stimulates the reproductive axis. Taking an *in vitro* approach, we determined whether kisspeptin directly regulates NPY mRNA expression and secretion in the mHypoE-38 and mHypoE-42 neurons.

Previously, our lab has characterized the murine-derived, embryonic, NPY-secreting cell lines mHypoE-38 and mHypoE-42 (34). These studies identified the mHypoE-38 cells as a putative reproductive NPY neuron because long-term estrogen exposure resulted in a 4-fold increase in NPY mRNA expression that is postulated to be involved in the GnRH reproductive hormone surge. In contrast, the mHypoE-42 cells were identified as a feeding-related NPY neuron because treatment with estrogen steadily repressed NPY mRNA expression to potentially exert anorexigenic effects. A subsequent study in our lab also found that conditioned media containing NPY synthesized from the mHypoE-38 cells significantly stimulated GnRH mRNA expression in the GT1-7 cell line, a GnRH neuronal model (35). We further characterized the mHypoE-38 and mHypoE-42 cell lines in the present study. Importantly, both lines demonstrated the presence of Kiss1r mRNA and thus represent natural targets of kisspeptin. We therefore examined the effect of kisspeptin on NPY mRNA expression and secretion in both cell lines. Treatment with kisspeptin revealed an approximately 70% induction in NPY mRNA expression after 24 h, and an approximately 30% increase in NPY secretion after 1 h in the mHypoE-38 neurons. Kisspeptin did not regulate NPY mRNA expression or secretion in the mHypoE-42 neurons. This effect of kisspeptin in the mHypoE-38 cells corroborates our previous characterization of the cell line as a reproductive NPY neuron. Interestingly, our kisspeptin-mediated increase in NPY secretion from the mHypoE-38 neurons secreted a comparable amount of NPY (~1.1 ng/ml) that was previously demonstrated to directly stimulate GnRH transcription in GT1-7 neurons (35). To our knowledge, these findings are the first to indicate that kisspeptin can directly stimulate NPY neurons, potentially resulting in NPY activation of GnRH neurons.

In support of our findings, one recent study demonstrated close apposition of kisspeptin fibers and NPY neurons using double-label fluorescent immunohistochemistry (36). This study further showed that infusion of kisspeptin into the third ventricle of ovariectomized ewes results in an approximate 2-fold increase in NPY mRNA

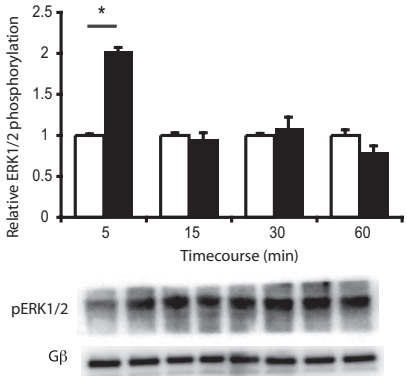


FIG. 5. mHypoE-42 neurons were serum starved for 4 h before treatment with kisspeptin (10 nM) over a 1-h time course. At the indicated timepoints, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phosphospecific antibodies directed against ERK1/2. Results shown are relative to control protein Gβ (set to 1.0) and are expressed as mean ± SEM (n = three independent experiments). *, $P < 0.05$. *White bar*, Vehicle control; *black bar*, kisspeptin treatment.

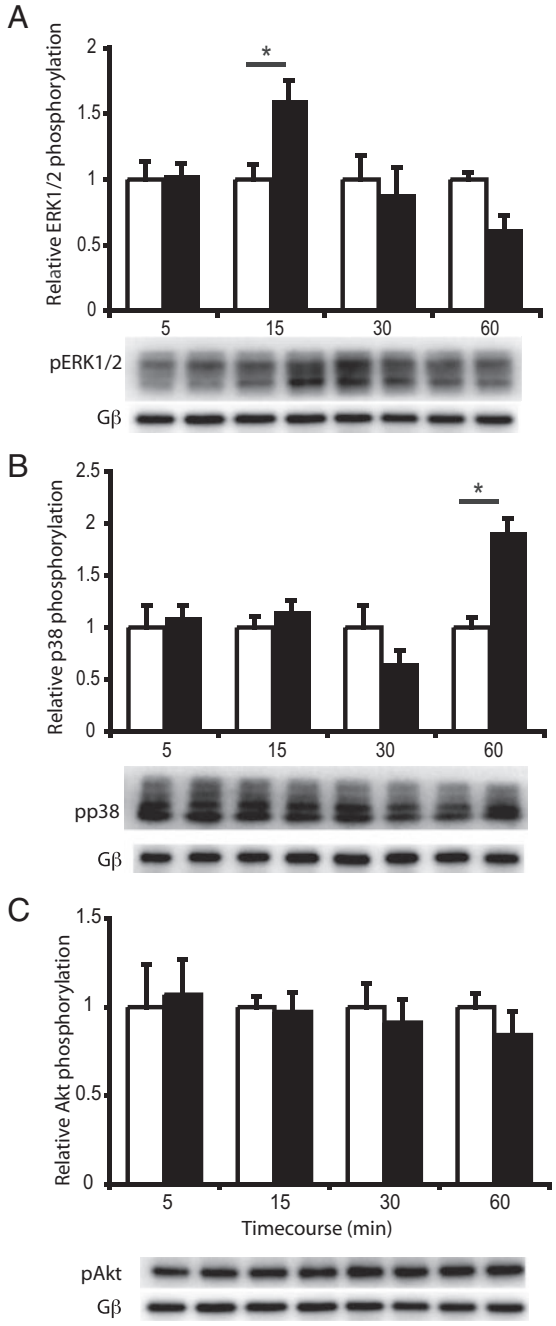


FIG. 6. mHypoE-38 neurons were serum starved for 4 h before treatment with kisspeptin (10 nM) over a 1-h time course. At the indicated timepoints, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phosphospecific antibodies directed against ERK1/2 (A), p38 (B), and Akt (C). Results shown are relative to control protein Gβ (set to 1.0) and expressed as mean ± SEM (n = three independent experiments). *, $P < 0.05$. *White bar*, Vehicle control; *black bar*, kisspeptin treatment.

expression within the arcuate nucleus, in terms of both level of expression per cell and number of cells detected. These ewes were also found to have an associated increase in LH levels. Although these findings in combination with the present study are strongly supportive of a kisspeptin-NPY pathway, the exact physiological implications of this pathway on the gonadotropin axis remain to be determined. Current literature suggests that a substantial part of the actions of kisspeptin on GnRH are conducted via direct activation of GnRH neurons because the majority of GnRH neurons (~70%) express Kiss1r (7) and kisspeptin is the most potent stimulator of gonadotropin release known to date (37). Likewise, NPY is less potent than kisspeptin in stimulating LH release (21) and may have inhibitory effects on the gonadotropic axis under certain conditions (27–32). Therefore, we suggest that the proposed indirect actions of kisspeptin on GnRH neurons via afferent NPY neurons may play a more complementary role to the direct stimulatory effect of kisspeptin on GnRH release. Nonetheless, future studies examining the gonadotropin response to kisspeptin administration after blocking the NPY response (*e.g.* with a NPY antagonist) will be highly important in establishing the physiological relevance of this pathway.

Interestingly, a reciprocal action of NPY on Kiss1 gene expression has been documented. Correlational studies have shown that NPY knockout mice have significantly reduced hypothalamic Kiss1 mRNA levels (38), whereas fasting-induced increases in hypothalamic NPY mRNA levels are associated with an estrogen-dependent decrease in Kiss1 mRNA expression in the anteroventral periventricular nucleus (39). To establish a direct effect of NPY on kisspeptin synthesis, one study found that treatment of NPY in a hypothalamic cell line resulted in an increase in Kiss1 mRNA expression (38). Furthermore, NPY fibers have been found in close contact with kisspeptin neurons using double-label immunohistochemistry (36). Although further studies examining the direct effect of NPY on kisspeptin and kisspeptin on NPY are required, it seems

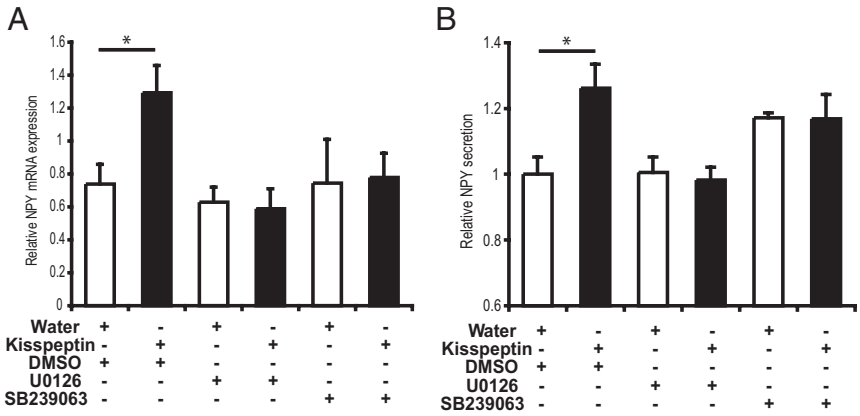


FIG. 7. A, mHypoE-38 cells were pretreated with U0126 or SB239063 (25 μM) for 1 h and then cotreated with kisspeptin (10 nM) for 24 h. NPY mRNA expression was determined using real-time RT-PCR, and levels were normalized to histone H3 (n = three to eight independent experiments). B, mHypoE-38 cells were pretreated with U0126 or SB239063 (25 μM) for 1 h and then cotreated with kisspeptin (10 nM) for 1 h. Concentration of NPY in the media was measured using an EIA. NPY secretion levels were set to 1.0 (n = three to eight independent experiments). Data are shown as mean ± SEM. *, $P < 0.05$.

likely that a reciprocal action exists between these two reproductive neuropeptides, thus adding another layer of control in the precise regulation of GnRH neurons.

Molecular studies have reported that kisspeptin activates the G protein $G_{q/11}$, resulting in activation of phospholipase C as well as the MAPK and PI3K/Akt pathways (15, 40–47). However, these studies were completed in either heterologous cell models transfected with Kiss1r (42–47) or the GT1-7 and GN11 GnRH neuronal cell models (15). Alternatively, we used the NPY-secreting mHypoE-38 neurons that endogenously express Kiss1r to examine the pathways activated by kisspeptin. Using Western blot analysis, we found that kisspeptin treatment significantly increased phosphorylation of ERK1/2 after 15 min and p38 after 1 h in the mHypoE-38 cells. Kisspeptin did not significantly change the phosphorylation of Akt in these cells. These results are in agreement with those initially reported for Kiss1/Kiss1r signaling using heterologous cell models. However, our data also highlight a potential divergence of Kiss1/Kiss1r signaling in NPY and GnRH neurons. Kisspeptin stimulates ERK1/2 and p38 in both NPY and GnRH neurons; however, kisspeptin also activates Akt specifically in GnRH neurons (15).

Additional studies further investigated the specific intracellular signaling cascades responsible for the stimulatory effects of kisspeptin on GnRH neurons. Using pharmacological inhibitors against key pathways involved in Kiss/Kiss1r signaling, these studies have confirmed that the MAPK, particularly ERK1/2 and p38, and PI3K/Akt pathways mediate kisspeptin-stimulated GnRH release in both *in vitro* GnRH cell models (15) and *ex vivo* hypothalamic explants (48). In the present study, we alternatively examined the signaling events involved in the regulation of NPY synthesis and secretion by kisspeptin. We found that the 24-h kisspeptin-mediated induction of NPY mRNA expression was significantly attenuated by the MAPK inhibitor U0126 and the p38 inhibitor SB239063. We further assessed the role of these pathways in kisspeptin-stimulated NPY secretion. Similarly, U0126 and SB239063 abolished the 1-h kisspeptin-mediated increase in NPY secretion. The PI3K inhibitor LY294002 did not significantly reduce kisspeptin-induction of NPY mRNA expression or secretion (data not shown). Taken together, these results demonstrate that the ERK1/2 and p38 MAPK pathways, but not the PI3K pathway, are essential in the kisspeptin-mediated increase in NPY synthesis and secretion, which is in contrast to the pathways mediating kisspeptin stimulation of GnRH. As a result, we postulate that kisspeptin can regulate the reproductive axis both directly at the GnRH neuron and indirectly at the NPY neuron through integration of intrinsically different pathways.

The mHypoE-38 and -42 cells were found to express AgRP, ghrelin, and NT, which are involved in the control of both feeding and reproduction (49–54). We therefore investigated the possibility that kisspeptin regulates the expression of these neuropeptides in our putative feeding-related NPY neuron, mHypoE-42, and reproductive NPY neuron, mHypoE-38. We also studied the effect of kisspeptin on Kiss1r mRNA expression to determine the ability of kisspeptin to regulate its own receptor. Both cell lines were treated with kisspeptin over a 24-h time course, and it was found that kisspeptin did not alter AgRP, ghrelin, NT, or Kiss1r mRNA expression in the mHypoE-42 cells, or AgRP, ghrelin, or Kiss1r mRNA expression in the mHypoE-38 cells. Because kisspeptin was not found to regulate any of these genes including NPY in the mHypoE-42 cells, we tested the functionality of the Kiss1r receptor in these cells. We found that phosphorylation of ERK1/2 was significantly increased with kisspeptin exposure, indicating that Kiss1r is indeed functional in this cell line. The downstream effect of Kiss1r activation in this cell line, however, has yet to be determined.

Several studies have reported expression of Kiss1r in non-GnRH neurons of the hypothalamus (7, 16); however, neither the identity nor the function of these hypothalamic Kiss1r-expressing neurons has been determined as yet. We demonstrate that NPY neurons from the mouse hypothalamus express Kiss1r, and kisspeptin directly increases NPY mRNA expression and secretion in an NPY-

secreting cell line via the ERK1/2 and p38 MAPK signaling pathways. Thus, we provide evidence that kisspeptin directly regulates NPY neurons and suggest that the indirect effects of kisspeptin on the GnRH neuron may occur at least partially through activation of afferent NPY neurons. Our study provides significant insight into the precise mechanisms underlying kisspeptin regulation of the reproductive axis. Delineation of these mechanisms is important because kisspeptin is widely being considered as a promising new treatment for infertility disorders (55).

Acknowledgments

We acknowledge funding from the Canadian Institutes for Health Research, Canada Foundation for Innovation, and the Canada Research Chairs Program (to D.D.B.).

Address all correspondence and requests for reprints to: Denise D. Belsham, Ph.D., Department of Physiology, University of Toronto, Medical Sciences Building 3247A, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8. E-mail: d.belsham@utoronto.ca.

G.L.K. was supported by a Natural Sciences and Engineering Research Council of Canada Studentship and an Ontario Graduate Scholarship.

Disclosure Summary: The authors have nothing to declare.

References

1. **de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E** 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100:10972–10976
2. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley Jr WF, Aparicio SA, Colledge WH** 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614–1627
3. **Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL** 2003 The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 312:1357–1363
4. **d'Anglemont de Tassigny X, Fagg LA, Dixon JP, Day K, Leitch HG, Hendrick AG, Zahn D, Franceschini I, Caraty A, Carlton MB, Aparicio SA, Colledge WH** 2007 Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc Natl Acad Sci USA 104:10714–10719
5. **Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K** 2005 Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146:4431–4436
6. **Clarkson J, Herbison AE** 2006 Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 147:5817–5825
7. **Herbison AE, de Tassigny X, Doran J, Colledge WH** 2010 Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons. Endocrinology 151:312–321
8. **Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA** 2005 Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc Natl Acad Sci USA 102:1761–1766
9. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA** 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073–4077
10. **Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2004 Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145: 4565–4574
11. **Navarro VM, Castellano JM, Fernández-Fernández R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2005 Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. Endocrinology 146:1689–1697
12. **Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR** 2005 Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab 90: 6609–6615
13. **Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE** 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25:11349–11356
14. **Liu X, Lee K, Herbison AE** 2008 Kisspeptin excites gonadotropin-releasing hormone neurons through a phospholipase C/calcium-dependent pathway regulating multiple ion channels. Endocrinology 149:4605–4614
15. **Novaira HJ, Ng Y, Wolfe A, Radovick S** 2009 Kisspeptin increases GnRH mRNA expression and secretion in GnRH secreting neuronal cell lines. Mol Cell Endocrinol 311:126–134
16. **Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-Nguyen AT, George SR, O'Dowd BF** 1999 Discovery of a receptor related to the galanin receptors. FEBS Lett 446:103–107
17. **Pielecka-Fortuna J, Chu Z, Moenter SM** 2008 Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. Endocrinology 149:1979–1986
18. **Li C, Chen P, Smith MS** 1999 Morphological evidence for direct interaction between arcuate nucleus neuropeptide Y (NPY) neurons and gonadotropin-releasing hormone neurons and the possible involvement of NPY Y1 receptors. Endocrinology 140:5382–5390
19. **Dudás B, Mihály A, Merchenthaler I** 2000 Topography and associations of luteinizing hormone-releasing hormone and neuropeptide Y-immunoreactive neuronal systems in the human diencephalon. J Comp Neurol 427:593–603
20. **Kalra SP, Dube MG, Sahu A, Phelps CP, Kalra PS** 1991 Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. Proc Natl Acad Sci USA 88:10931–10935
21. **Bauer-Dantoin AC, McDonald JK, Levine JE** 1992 Neuropeptide Y potentiates luteinizing hormone (LH)-releasing hormone-induced LH secretion only under conditions leading to preovulatory LH surges. Endocrinology 131:2946–2952
22. **Crowley WR, Hassid A, Kalra SP** 1987 Neuropeptide Y enhances the release of luteinizing hormone (LH) induced by LH-releasing hormone. Endocrinology 120:941–945
23. **Advis JP, Klein J, Kuljis RO, Sarkar DK, McDonald JM, Conover CA** 2003 Regulation of gonadotropin releasing hormone release by

neuropeptide Y at the median eminence during the preovulatory period in ewes. Neuroendocrinology 77:246–257
24. **Besecke LM, Wolfe AM, Pierce ME, Takahashi JS, Levine JE** 1994 Neuropeptide Y stimulates luteinizing hormone-releasing hormone release from superfused hypothalamic GT1-7 cells. Endocrinology 135:1621–1627
25. **Xu M, Hill JW, Levine JE** 2000 Attenuation of luteinizing hormone surges in neuropeptide Y knockout mice. Neuroendocrinology 72: 263–271
26. **Sutton SW, Toyama TT, Otto S, Plotsky PM** 1988 Evidence that neuropeptide Y (NPY) released into the hypophysial-portal circulation participates in priming gonadotropes to the effects of gonadotropin releasing hormone (GnRH). Endocrinology 123:1208–1210
27. **Gonzales C, Voirol MJ, Giacomini M, Gaillard RC, Pedrazzini T, Pralong FP** 2004 The neuropeptide Y Y1 receptor mediates NPY-induced inhibition of the gonadotrope axis under poor metabolic conditions. FASEB J 18:137–139
28. **Pierroz DD, Gruaz NM, d'Alièves V, Aubert ML** 1995 Chronic administration of neuropeptide Y into the lateral ventricle starting at 30 days of life delays sexual maturation in the female rat. Neuroendocrinology 61:293–300
29. **McDonald JK, Lumpkin MD, DePaolo LV** 1989 Neuropeptide-Y suppresses pulsatile secretion of luteinizing hormone in ovariectomized rats: possible site of action. Endocrinology 125:186–191
30. **Kaynard AH, Pau KY, Hess DL, Spies HG** 1990 Third-ventricular infusion of neuropeptide Y suppresses luteinizing hormone secretion in ovariectomized rhesus macaques. Endocrinology 127:2437–2444
31. **Catzeflis C, Pierroz DD, Rohner-Jeanrenaud F, Rivier JE, Sizonenko PC, Aubert ML** 1993 Neuropeptide Y administered chronically into the lateral ventricle profoundly inhibits both the gonadotropic and the somatotropic axis in intact adult female rats. Endocrinology 132:224–234
32. **Khorram O, Pau KY, Spies HG** 1987 Bimodal effects of neuropeptide Y on hypothalamic release of gonadotropin-releasing hormone in conscious rabbits. Neuroendocrinology 45:290–297
33. **Belsham DD, Cai F, Cui H, Smukler SR, Salapatek AM, Shkreta L** 2004 Generation of a phenotypic array of hypothalamic neuronal cell models to study complex neuroendocrine disorders. Endocrinology 145:393–400
34. **Titolo D, Cai F, Belsham DD** 2006 Coordinate regulation of neuropeptide Y and agouti-related peptide gene expression by estrogen depends on the ratio of estrogen receptor (ER) α to ERβ in clonal hypothalamic neurons. Mol Endocrinol 20:2080–2092
35. **Dhillon SS, Gingerich S, Belsham DD** 2009 Neuropeptide Y induces gonadotropin-releasing hormone gene expression directly and through conditioned medium from mHypoE-38 NPY neurons. Regul Pept 156:96–103
36. **Backholer K, Smith JT, Rao A, Pereira A, Iqbal J, Ogawa S, Li Q, Clarke IJ** 2010 Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells. Endocrinology 151:2233–2243
37. **Navarro VM, Castellano JM, Fernández-Fernández R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2005 Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology 146: 156–163
38. **Luque RM, Kineman RD, Tena-Sempere M** 2007 Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. Endocrinology 148:4601–4611
39. **Kalamatianos T, Grimshaw SE, Poorun R, Hahn JD, Coen CW** 2008 Fasting reduces KiSS-1 expression in the anteroventral periventricular nucleus (AVPV): effects of fasting on the expression of KiSS-1 and neuropeptide Y in the AVPV or arcuate nucleus of female rats. J Neuroendocrinol 20:1089–1097
40. **Castaño JP, Martínez-Fuentes AJ, Gutiérrez-Pascual E, Vaudry H, Tena-Sempere M, Malagón MM** 2009 Intracellular signaling pathways activated by kisspeptins through GPR54: do multiple signals underlie function diversity? Peptides 30:10–15
41. **Colledge WH** 2009 Kisspeptins and GnRH neuronal signalling. Trends Endocrinol Metab 20:115–121
42. **Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brézillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G, Parmentier M** 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem 276:34631–34636
43. **Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekeres PG, Sarau HM, Chambers JK, Murdock P, Steplewski K, Shabon U, Miller JE, Middleton SE, Darker JG, Larminie CG, Wilson S, Bergsma DJ, Emson P, Faull R, Philpott KL, Harrison DC** 2001 AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem 276:28969–28975
44. **Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M** 2001 Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411:613–617
45. **Ringel MD, Hardy E, Bernet VJ, Burch HB, Schuppert F, Burman KD, Saji M** 2002 Metastin receptor is overexpressed in papillary thyroid cancer and activates MAP kinase in thyroid cancer cells. J Clin Endocrinol Metab 87:2399
46. **Stafford LJ, Xia C, Ma W, Cai Y, Liu M** 2002 Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor. Cancer Res 62:5399–5404
47. **Stathatos N, Bourdeau I, Espinosa AV, Saji M, Vasko VV, Burman KD, Stratakis CA, Ringel MD** 2005 KiSS-1/G protein-coupled receptor 54 metastasis suppressor pathway increases myocyte-enriched calcineurin interacting protein 1 expression and chronically inhibits calcineurin activity. J Clin Endocrinol Metab 90:5432–5440
48. **Castellano JM, Navarro VM, Fernandez-Fernandez R, Castano JP, Malagon MM, Aguilar E, Dieguez C, Magni P, Pinilla L, Tena-Sempere M** 2006 Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat. Mol Cell Endocrinol 257–258:75–83
49. **Beck B** 2000 Neuropeptides and obesity. Nutrition 16:916–923
50. **Castañeda TR, Tong J, Datta R, Culler M, Tschöp MH** 2010 Ghrelin in the regulation of body weight and metabolism. Front Neuroendocrinol 31:44–60
51. **Morton GJ, Schwartz MW** 2001 The NPY/AgRP neuron and energy homeostasis. Int J Obes Relat Metab Disord 25(Suppl 5):S56–S62
52. **RostèneWH, Alexander MJ** 1997 Neurotensin and neuroendocrine regulation. Front Neuroendocrinol 18:115–173
53. **Stanley SA, Small CJ, Kim MS, Heath MM, Seal LJ, Russell SH, Ghatei MA, Bloom SR** 1999 Agouti-related peptide (Agrp) stimulates the hypothalamo pituitary gonadal axis *in vivo*, *in vitro* in male rats. Endocrinology 140:5459–5462
54. **Tena-Sempere M** 2008 Ghrelin as a pleotrophic modulator of gonadal function and reproduction. Nat Clin Pract Endocrinol Metab 4:666–674
55. **Jayasena CN, Dhillo WS** 2009 Kisspeptin offers a novel therapeutic target in reproduction. Curr Opin Investig Drugs 10:311–318