Endocr (2011) 39:220–228
DOI 10.1007/s12020-011-9439-4

ORIGINAL ARTICLE

Effects of kisspeptin on parameters of the HPA axis

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Received: 4 February 2011 / Accepted: 5 February 2011 / Published online: 9 March 2011

Abstract The hypothalamo–pituitary–adrenal (HPA) and hypothalamo–pituitary–gonadal (HPG) axes have an intricate cross talk that results in the inhibition of reproductive functions during periods of chronic physiological or psychological stress. Recent studies have shown that kisspeptin neurons have projections to many non-reproductive areas of the brain including the paraventricular nucleus (PVN) of the hypothalamus, thereby providing evidence of an anatomical framework for kisspeptin to regulate the HPA axis. In this study, we tested as to whether kisspeptin modulates the HPA axis at three potential levels of regulation: (1) transcription of stress-related genes CRH, AVP, and oxytocin (OXY); (2) release of neuropeptides from PVN-derived neuronal cells via mobilization of intracellular calcium stores; and (3) in vivo regulation of the HPA axis under basal and stress-induced conditions in adult male rats. Overall, our data showed that kisspeptin did not alter basal, or stress-induced HPA axis activity (plasma corticosterone (CORT) and adrenocorticotropin hormone (ACTH)) in adult male rats and had modest, yet significant effects on CRH, AVP, and OXY gene expressions.

Keywords HPA · Stress · Kisspeptin · AVP · CRH

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Introduction

The hypothalamo–pituitary–adrenal (HPA) and hypothalamo–pituitary–gonadal (HPG) axes are intimately connected, as chronic physiological stress and high-circulating levels of glucocorticoids typically inhibit reproductive functions [1–3]. Glucocorticoids negatively regulate gonadotropin-releasing hormone (GnRH) promoter activity and decrease GnRH receptor transcription [4], leading to a decreased gonadal function and lower levels of circulating gonadal steroid hormones. Gonadal steroid hormones, in turn, regulate physiological parameters of the HPA axis. For instance, testosterone (T) in males decreases circulating levels of glucocorticoids [5], while 17β-estradiol (E2) has the opposite effect in females [6]. Gonadal steroid hormones also regulate the central expression of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP), which are critical upstream activators of the HPA axis [7–10]. Thus, the physiological "cross talk" between these two systems conveys important homeostatic information to ensure optimal reproductive success.

Recent studies have implicated the neurohormone kisspeptin as a primary upstream regulator of GnRH neurons and the HPG axis. Kisspeptin is a 54-amino acid neuropeptide that is secreted from neurons in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC; [11, 12]). Kisspeptin targets neurons in the basal hypothalamus to stimulate the release of GnRH through its cognate receptor *Kiss1r* (formerly called GPR54). The end result of this action is a dramatic increase in circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and subsequent stimulation of the reproductive organs [13–17]. Kisspeptin and its receptor *Kiss1r* are abundantly expressed in many areas of the brain, including

the paraventricular nucleus (PVN [18]), which is key brain region associated with modulation of the stress response. Although a role for kisspeptin in these neuronal systems has not yet been studied in detail, these data provide an anatomical framework for the possibility that, in addition to its effects on the HPG axis, kisspeptin might also play a role in the regulation of the HPA axis.

Kiss-1 and *Kiss1r* gene expression increase during development, reaching peak levels coincident with pubertal onset [15, 19]. Moreover, adult humans with mutations in the *KISS1R* gene, and mice with a targeted deletion of *Kiss1r* (*Kiss1r*KO), have undeveloped gonads, lack mature gametes, and have low-circulating gonadal steroid hormone levels [20–22]. Furthermore, mice lacking a functional *Kiss-1* gene also do not achieve sexual maturation [21]. Together, these data demonstrate that kisspeptin signaling is an absolute requirement for normal pubertal development. Puberty is also a critical developmental period for maturation of the HPA axis, raising the possibility that kisspeptin might serve a dual role in regulating both the HPG and HPA systems. This possibility would provide a novel mechanism for cross talk between the HPA and HPG axes and would indicate an early interaction between the two pathways during development.

We hypothesized that kisspeptin would decrease HPA axis reactivity by lowering stress-induced corticosterone (CORT) and transcription of stress-related genes CRH, AVP, and oxytocin (OXY). In this study, we tested whether kisspeptin modulates the HPA axis at three potential levels of regulation: (1) expression of stress-related genes CRH, AVP, and OXY; (2) release of neuropeptides from PVN-derived neuronal cells via mobilization of intracellular calcium stores; and (3) in vivo regulation of the HPA axis under basal and stress-induced conditions.

Materials and methods

Cell culture

All the cell lines used in these studies were verified to be free of mycoplasma contamination (MycoSensor QPCR, Stratagene/Agilent Technologies). The following tumorigenic cell lines were used: H32 (derived from rat PVN, generously provided by Dr. Joachim Speiss, University of Hawaii) and IVB (derived from rat PVN, generously provided by Dr. John Kaskow, University of Cincinnati). These cell lines were chosen based on their PVN origin. Cells were cultured with Dulbecco's modified essential medium (DMEM) containing 4.5 g/l glucose and L-glutamine (Cellgro, Manassas, VA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), penicillin, streptomycin, ampicillin, and gentamycin. Cells were grown to 70% confluency and used within 10 passages for all experiments.

Peptides

Kiss-1 (110–119) Amide Mouse (Phoenix Pharmaceuticals, Catalog # 048-65) was resuspended in 0.09% saline (in vivo studies) or 1× PBS. For cell culture studies, kisspeptin was serially diluted in cell culture media at 0.001 μM, 0.01 μM, 0.1 μM, and 1.0 μM concentrations. Cells were treated at each dose for 4 or 8 h.

Quantitative RT-PCR

RNA isolation

H32 and IVB cells were plated at a density of 2.0×10^5 cells/well in a six-well plate. Cells were allowed to grow in regular media containing 10% FBS for 24–48 h until 70–80% confluent. On the day of treatment, cells were treated with vehicle or 0.01, 0.1, and 1.0 μM kisspeptin for 4 or 8 h. All the treatments were done in replicates of six wells. Cells were washed once with cold PBS, lysed with Trizol reagent, and total RNA isolated according to manufacturer's instructions (Invitrogen Inc., Carlsbad, CA). Following isolation, genomic DNA contamination was removed using DNAfree (Stratagene, a division of Agilent Corp., La Jolla, CA) according to manufacturer's instructions. Quantification of total RNA was performed using a Nanodrop spectrophotometer, and samples with an OD 260:280 of 1.7–1.9 were used for subsequent reverse transcription assays.

cDNA synthesis

Total RNA (1 μg) was combined with 0.5 μg oligo d(T), heated to 65°C and rapidly cooled with ice. The RNA–primer mix was combined with M-MLV buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.5 mM dNTP, and 0.5 mM M-MLV reverse transcriptase (Invitrogen Inc., Carlsbad, CA). Reverse transcriptase reaction was performed by incubating for 10 min at room temp, 50 min at 42°C, and then 95°C for 5 min to terminate the reaction.

qRT-PCR

qPCR was performed using FastStart DNA Master SYBR Green I according to manufacturer's instructions (Roche Molecular Biomedical, Indianapolis, IN). Master mix containing $MgCl_2$, SYBR Green, and primer pairs

(0.25 μM) were aliquoted into 96-well plates followed by the addition of 1/20th of the reverse transcription reaction (cDNA). No template controls received DNA-free water of the same volume. All the cDNA samples were tested in triplicate within an assay, and each experiment was repeated three times. Real-time PCR reactions were carried out using the Eppendorf Realplex thermocycler with the following conditions: 95°C for 10 min, 40 repeated cycles including denature (95°C), annealing (60°C), and extension (72°C) with fluorescence detection at the end of each 72°C step, and then the samples were melted under continuous fluorescence detection to 95°C. PCR products were resolved on a 2% agarose gel and compared with a DNA ladder of known size (Fisher Scientific, Exactgene 50 bp ladder) to confirm product size, and to verify specificity, the products were subjected to a thermal melting curve analysis to determine whether the Tm of the product was consistent with the calculated theoretical Tm based on sequence. Intron-spanning primer sequences are as follows: CRH sequence—5-CTGGGGAACCTCAACAGAAG; 3-GGTGGAAGGTGAGATCCAGA; AVP sequence—5-CGCAGTGCCCACCTATGCTC; 3-AGGAAGCAGCCCAGCTCGTC; OXY sequence—5-CTTGGCCTACTGGGTCTGAC; 3-GGGCAGGTAGTTCTCCTCCT. All the samples were normalized to the constitutively expressed hypoxanthine phosphoribosyl transferase 1 (HPRT) housekeeping gene. Fold change was calculated using the conventional formula $2^{(-(\Delta\Delta CT))}$.

Animals

Adult male Sprague–Dawley rats ($N = 40$) were purchased from Charles River Laboratories, Wilmington, MA. Animals were allowed to acclimate to the new environment for 7 days and then handled 5 min once/day for 7 days. Animals were housed two per cage. Animals were allowed free access to food and water, and all the animal studies were approved by the Institutional Animal Care and Use Committee at Loyola University Chicago, IACUC approval 2007048.

Treatment

Rats were given an intraperitoneal (i.p.) injection of saline ($n = 20$) or 0.13 μg/μl (25 μg/kg in 100 μl saline) kisspeptin ($n = 20$). Following injection, one rat within the cage was returned to the home cage, while the cagemate was subjected to a 30-min restraint stress in a plastic rodent restraint tube. After 30 min, rats were killed by rapid decapitation. Blood was collected into heparinized tubes, and brains were rapidly frozen in 2-methyl butane.

Radioimmunoassay

Trunk blood taken from adult male rats was centrifuged at 3,000 rpm at 4°C for 20 min. Plasma was extracted and stored at −20°C until processed by RIA for CORT, ACTH, and LH.

Corticosterone (CORT) RIA

First, 10 μl of sample plasma was added to 240 μl 0.01 M PBS and then heated to 65°C for 1 h. Total counts comprising 200 μl of 0.1% gel PBS and 100 μl of tracer (^{3}H-CORT Amersham). Non-specific-binding controls (NSB) comprising 200 μl of 0.1% gel PBS and 100 μl of tracer. Corticosterone standards were added to 13 mm × 100 mm borosilicate glass tubes. Concentrations of corticosterone standards were: 5, 10, 20, 30, 40, 50, 75, 100, 200, and 500 pg. Then, 100 μl 0.1% gel PBS was added to each standard tube. Rabbit anti-CORT (MP Biomedicals #07-120016) was reconstituted with 1 ml of deionized water and then diluted with 9 ml of 0.1% gel PBS. ^{3}H-CORT was diluted to 10,000–12,000 cpm/tube. 100 μl of ^{3}H-CORT was added to each tube. Borosilicate tubes were vortexed, covered with aluminum foil and incubated at 4°C overnight. On day 2, antibody-bound hormone was precipitated by the addition of 100 μl of 0.5% gel PBS and 1.0 ml of Dextran-coated Charcoal (DCC). Supernatant was collected into scintillations vials and radioactivity as counts per minute (cpm) was counted using a scintillation counter.

Adrenocorticotropin hormone (ACTH) RIA

Human ACTH standards (Bachem) were added to plastic 12 × 75 tubes. Concentrations of ACTH standards were: 0.2, 1, 2, 5, 10, 20, 50, and 100 pg. 50 μl of untreated rat samples, 20 μl of non-restraint rat samples, 10 μl of restraint rat samples were pipetted respectively into triplicate tubes. BSA buffer was added to standard and samples tubes to make up the volume up to 100 μl. 100 μl of rabbit anti-ACTH antibody (IgG Corp) was added to all the tubes except total and non-specific binding controls. Tubes were gently shaken, covered with foil, and placed into a 4°C fridge for 24 h. ^{125}I-ACTH (Diasorin) was diluted to 2,000 cpm/tube. 100 μl of ^{125}I-ACTH was added to all tubes and incubated at 4°C for 48 h. 100 μl of ant-rabbit IgG antibody (CalBiochem), and 100 μl of diluted normal rabbit serum (GibcoBRL) were added to all the tubes except totals. Tubes were vortexed and incubated at 4°C for 16 h. 500 μl of cold PBS buffer was added to all the tubes except totals and centrifuged for 40 min at 3,000 rpm at 4°C. Supernatant was decanted and radioactivity counted using a gamma counter.

Luteinizing hormone (LH) RIA

Detection of pituitary LH was performed as described previously in the laboratory of Dr. Pei-San Tsai at the University of Colorado, Boulder [23] using rat LH RIA kits obtained from Dr. A. F. Parlow (NIH National Pituitary Program). For LH RIA, rLH-I10, rLH-RP3, and rLH-S11 were used as the iodination stock, RIA standard, and antibody, respectively. In brief, for RIA, 50 µl sample was mixed with 50 µl antiserum (1:20,000 dilution) and allowed to incubate for 20 h at room temperature. On day 2, 100 µl iodinated tracer (20,000 cpm/100 µl) was added to RIA tubes and allowed to incubate for an additional 20 h at room temperature. Antibody-bound hormone was precipitated by the addition of 1 ml 5% polyethylene glycol containing a cocktail of goat antirabbit IgG (1:1,000), normal rabbit serum (1:2,000), and normal horse serum (1:2,000), and separated from the unbound tracer by centrifugation at 2,000×*g*. Using this RIA, we found the displacement curves of all serially diluted plasma and pituitary samples to be parallel to the rat LH standard. Limits of detection were 0.56 ng/ml. Intra- and inter-assay coefficients of variation were reported previously [23].

Calcium assays

Cells (H32, and IVB) were seeded into 96-well black-sided, and clear bottom plates at 30,000 cells per well in the first three columns. Cells were allowed to grow for 24 h and then were washed twice with warm calcium-free PBS. Plates were read on BioTek Synergy HT with Gen5 software program at EX: 380/20, 340/11, and EM: 508/20 for 30 s. Cells were then incubated with 200 µl of Fura-2AM (Invitrogen) and Krebs (with calcium) solution (5 µl of Pluronic Acid, 5 µl Fura-2AM, 10 mg BSA, 10 ml Krebs) for 1 h at room temperature wrapped in foil. Cells were washed with Krebs and then incubated with Krebs for 30 min to unload the cells. After unloading, cells were washed with PBS. Program for reading and dispensing is as follows: (Plate mode) 2-min reading (380 and 340), dispensing 100 µl, shaking at medium for 5 s, effecting a delay for 5 s, and taking the read for 5 min. The cells were treated with vehicle (PBS), 0.5 µM ionomycin/thapsigargin, or 1 µM kisspeptin.

Statistical analysis

One way ANOVA was used to test the differences between treatment groups in the animal experiments and also between treatment groups in cell culture RT-PCR experiments. If significance was found using the one way ANOVA, the Tukey's Multiple Comparison Test was used. A P value of less than 0.05 was considered significant.

Results

Effects of kisspeptin on expression of AVP, CRH, and OXY in PVN derived hypothalamic cell lines

The hypothalamic PVN-derived cell lines, H32 and IVB, were used to determine whether kisspeptin alters the expression of the CRH, AVP, and/or OXY genes in vitro. Before kisspeptin treatment, the cell lines were tested for baseline expression of these genes, as well as the kisspeptin receptor (*Kiss1r*). Both cell lines endogenously expressed OXY, AVP, and *Kiss1r*, however the H32 cells did not express detectable levels CRH mRNA (data not shown). Thus, the H32 cells were used to determine kisspeptin effects on AVP and OXY, and the IVB cells were used to determine kisspeptin effects on CRH and OXY. Cells were seeded into six-well plates and grown for 24 h before kisspeptin treatment (0.01, 0.1, or 1.0 µM) for 4 or 8 h. In the H32 cells, kisspeptin significantly increased AVP mRNA expression at all doses tested after 8 h of incubation (Fig. 1a). Similarly, kisspeptin (0.1 µM) significantly increased OXY mRNA expression in these same cells (Fig. 1b). In the IVB cells, kisspeptin significantly decreased CRH expression at the highest dose (1.0 µM;

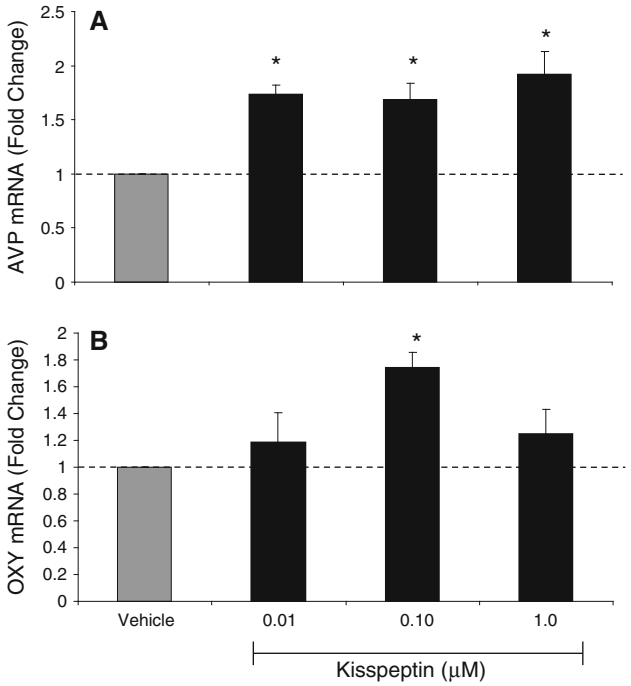

Fig. 1 Effects of kisspeptin on AVP and OXY expression in the PVN-derived neuronal cell line H32. Real time RT-PCR for AVP expression (**a**) and OXY expression (**b**) was performed on RNA isolated from H32 cells that were treated with vehicle, 0.01, 0.1, or 1.0 µM kisspeptin for 8 h. Data are represented as fold change in mRNA expression from vehicle-treated control ± SEM. The presence of an * denotes a statistical significance from vehicle-treated controls ($P < 0.05$)

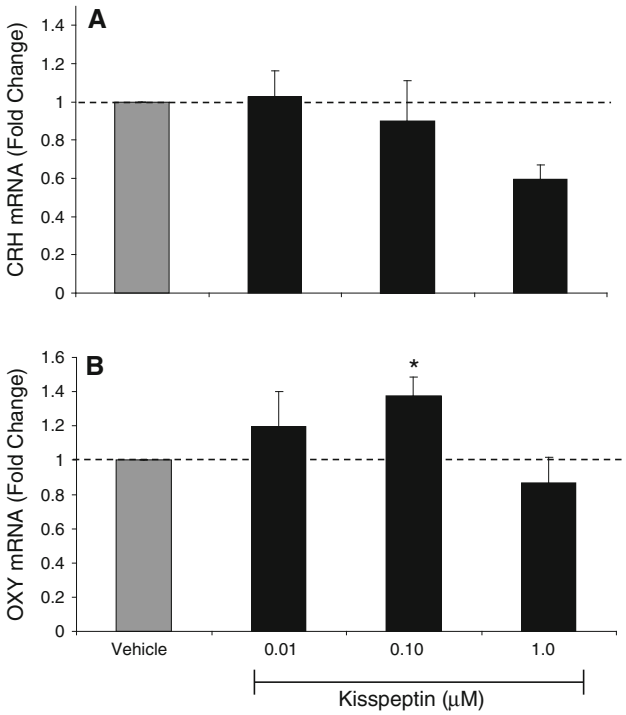

Fig. 2 Effects of kisspeptin on CRH and OXY expression in the PVN-derived neuronal cell line IVB. Real time RT-PCR for CRH expression (**a**) and OXY expression (**b**) was performed on RNA isolated from IVB cells that were treated with vehicle, 0.01, 0.1, or 1.0 μM kisspeptin for 8 h. Data are represented as fold change in mRNA expression from vehicle-treated control ± SEM. The presence of an * denotes a statistical significance from vehicle-treated controls ($P < 0.05$)

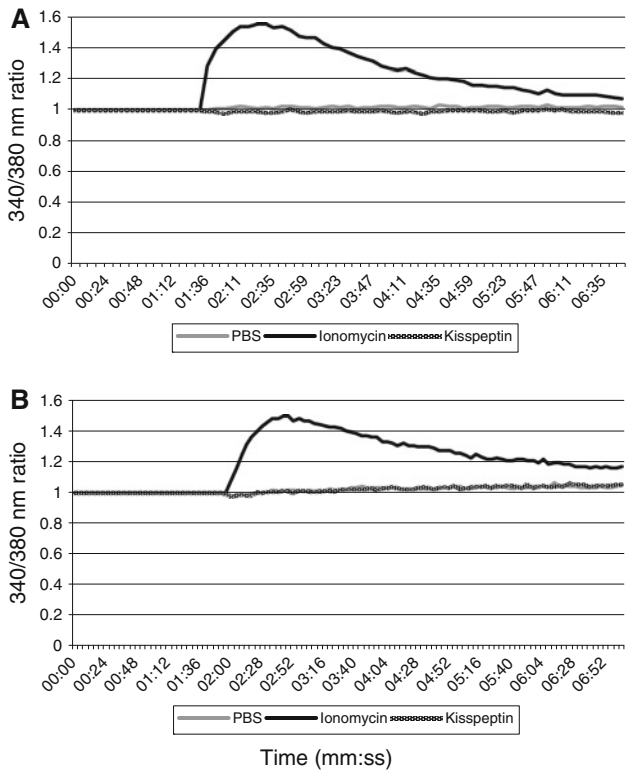

Fig. 3 Effects of kisspeptin on intracellular calcium mobilization in H32 and IVB neuronal cell lines. H32 (**a**) and IVB (**b**) cells were treated with PBS, ionomycin, or 1 μM kisspeptin. Data are represented as a normalized ratio of 340 nm/380 nm

Fig. 2a). Kisspeptin effects on OXY in IVB cells were similar to that observed in the H32 cells, with a significant increase in expression after 8 h of 0.1 μM kisspeptin treatment (Fig. 2b). There were no significant differences in either cell line for any of the genes after only 4 h of kisspeptin treatment (data not shown).

Mobilization of intracellular calcium stores in response to kisspeptin treatment in a variety of neuronal cell lines

The initial studies on kisspeptin identified its receptor, GPR54, to be part of the G_{q11} family of G protein-coupled receptors that activate phospholipase C and lead to mobilization of intracellular calcium stores [24, 25]. Further, intracellular calcium mobilization is one proposed mechanism mediating the observed kisspeptin-induced GnRH release from the hypothalamus [26, 27]. Therefore, to test whether kisspeptin could stimulate the release of intracellular calcium stores from PVN, we utilized the H32 and IVB cells. Kisspeptin treatment did not induce intracellular calcium mobilization in the H32 and IVB cells at any dose tested (Fig. 3). However, there was a robust response to treatment with ionomycin, a selective calcium ionophore that directly stimulates calcium release from intracellular stores, suggesting that these cells were capable of releasing calcium intracellularly (Fig. 3).

Effects of kisspeptin on plasma CORT, ACTH, and LH levels in adult male rats

These in vivo experiments were designed to determine whether kisspeptin could regulate the HPA axis under basal and/or stress-induced conditions. Adult male rats were given a single i.p. injection of saline or kisspeptin followed by a 30-min restraint stress or return to the home cage. As expected, 30 min of restraint stress significantly increased circulating levels of both CORT and ACTH (Fig. 4a, b, respectively). However, kisspeptin, when given alone, or with restraint stress, did not have any effect on plasma CORT or ACTH levels. As a positive control for the physiological efficacy of the kisspeptin, we also measured circulating levels of luteinizing hormone (LH). Consistent with previous reports, kisspeptin treatment induced a robust increase in plasma LH levels (Fig. 5). Moreover, the kisspeptin-induced increase in LH was unaffected by restraint stress (Fig. 5).

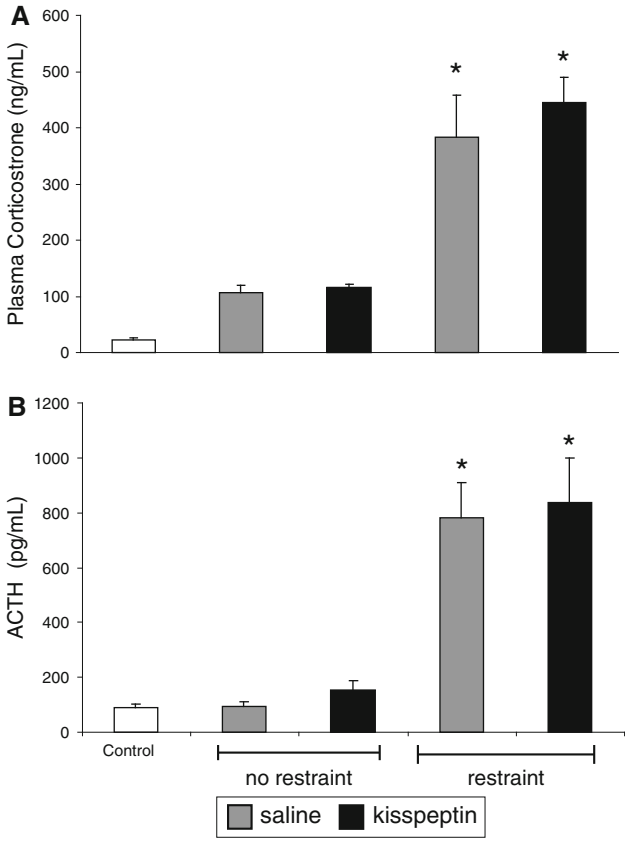

Fig. 4 Plasma CORT and ACTH levels in adult male rats after acute injection with kisspeptin. Adult male rats were given a single IP injection of saline or kisspeptin and then restraint stressed for 30 min or returned to their home cage. Plasma was analyzed by RIA for CORT (**a**) and ACTH (**b**) levels. The data are represented as mean ± SEM. The presence of an * denotes a statistical significance from vehicle-treated controls ($P < 0.05$)

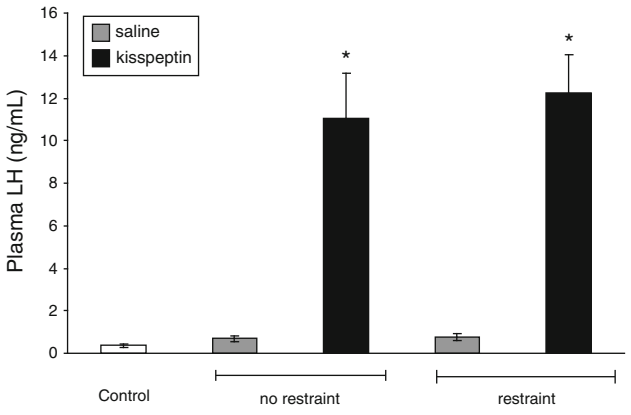

Fig. 5 Plasma LH levels in adult male rats after acute injection with kisspeptin. Adult male rats were given a single IP injection of saline or kisspeptin and were then restraint stressed for 30 min or returned to their home cage. Plasma was analyzed by RIA for LH levels. The data are represented as mean ± SEM. The presence of an * denotes a statistical significance from vehicle-treated controls ($P < 0.05$)

Discussion

In these experiments, we demonstrated that kisspeptin directly increased the gene expression of both AVP and OXY, while simultaneously decreasing CRH gene expression, in PVN-derived neuronal cells. However, these changes in gene expression were modest (1–2 fold changes) despite the fact that they were statistically significant. When coupled with the observations that kisspeptin had no effect on intracellular calcium release in PVN-derived cells and also had no effect on the stress response in vivo, it is logical to conclude that kisspeptin most likely does not play a major role in regulating HPA axis activity.

It is now well accepted that kisspeptin is a primary regulator of HPG axis activity [28]; however its role in other centrally regulated physiological processes remains unclear. The functions of the HPA and HPG axes are intimately connected, with physiological and/or psychological stressors adversely affecting reproductive function. Therefore, in these studies, our first aim was to determine whether kisspeptin regulated CRH, AVP, or OXY gene expression, all primary central mediators of HPA axis function. Our results showed a modest, yet statistically significant increase in AVP and OXY mRNA expressions following kisspeptin stimulation in the PVN-derived cell lines H32 and IVB (see Figs. 1, 2). Paradoxically, kisspeptin significantly decreased CRH gene expression in the IVB cells at the highest dose, suggesting that the effects of kisspeptin might differentially affect select regulatory components of the HPA axis. However, despite the statistically significant effect, it is unlikely that this dose of kisspeptin reflects physiological levels in neurons; therefore, a logical conclusion is that kisspeptin does not regulate CRH mRNA. *Kiss-1* neurons also project their axons to other AVP-expressing brain regions such as the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MeA) [18]. Both the BNST and MeA have been implicated in mediating social interactions, like pair bonding and paternal behaviors, as well as anxiety behavior [29–32]. Similarly, OXY has been associated with regulating pair bonding, anxiety, and stress-related behaviors, and OXY-containing neurons are frequently colocalized with neurons that express AVP [33–35]. Interestingly, AVP-expressing neurons from these limbic structures are known to project axons to the PVN, where it is thought that they provide primarily inhibitory inputs [7, 36]. Therefore, *Kiss-1* neurons may promote activation of the HPG axis, in part, by stimulating these inhibitory limbic pathways to the PVN, and thereby attenuating potential inhibitory signals from the HPA axis.

Based on our results from the PVN cell culture studies, we expected that in vivo *Kiss-1* peptide administration in adult male rats would alter circulating CORT and ACTH

levels. However, this was not the case. There are several possible explanations that may account for these observations. First, adult animals were chosen for this study due to the variability in HPA and HPG axes maturity observed in juvenile and peripubertal animals [37, 38]. However, recent reports have demonstrated that both *Kiss-1* and *Kiss1r* gene expressions are developmentally regulated, with peak expression in male rats occurring between 4 and 8 weeks of age [15, 19, 39]. Further, the gene expression profiles are also region specific, suggesting that kisspeptin action on the HPA axis might be confined to a particular developmental stage. Second, our choice to use males only in this study might have limited our findings. *Kiss-1* expression in the AVPV is sexually dimorphic, with higher levels of gene expression found in females compared with males [39]. While there have been no studies documenting a sex difference in kisspeptin fiber projections to the PVN, this possibility cannot be ruled out. In addition, circulating gonadal steroid hormones differentially affect the stress response in males and females, as testosterone has been shown to blunt the stress-induced increase in circulating CORT, and HPG function in females is more sensitive to the deleterious effects of chronic stress [5, 9, 40, 41]. Third, we measured CORT levels following a 30-min restraint stress paradigm which accurately assesses the immediate acute response to a stressor. However, chronic stress results in a different CORT profile with changes that occur in the timing for CORT to return to baseline following a stressor, as well as in habituation of the HPA axis to subsequent stressors [42]. It is possible that kisspeptin preferentially affects these aspects of the stress response rather than the immediate response to an acute stressor. Finally, as mentioned previously, kisspeptin might have been unable to cross the blood–brain barrier following our i.p. route of administration and therefore, acted directly at the level of the pituitary to increase LH. Taken together, these data highlight the complexity of kisspeptin action, and determining a direct effect of kisspeptin on the HPA axis in vivo requires further investigation. To our knowledge, there have been no other reports of kisspeptin regulating CORT or ACTH plasma levels. However, Kinsey-Jones et al. [43] showed that stress-induced plasma CORT decreased both *Kiss-1* and *Kiss1r* gene expression in the medial preoptic area and arcuate nucleus of adult female rats, providing further evidence of a possible regulatory link between kisspeptin and the HPA axis at the level of the CNS.

Kisspeptin has been shown to dramatically increase circulating levels of FSH and LH within 30 min [17, 44]. Our results are consistent with these previous reports, as kisspeptin administration significantly increased circulating LH levels in the adult male rats 30 min after injection. These data demonstrate that our kisspeptin preparation was biologically active; however, it does not rule out the possibility that it was acting directly at the level of the pituitary. Both *Kiss-1* and *Kiss1r* are expressed in rat pituitary, and kisspeptin has been shown to induce LH and growth hormone (GH) release from rat pituitary cells in vitro [45, 46]. Moreover, kisspeptin elicited GnRH release at the level of the median eminence in mediobasal hypothalamic explants [47].

Kisspeptin stimulates neuronal firing, through mobilization of intracellular calcium stores via PIP2 cleavage, and hence, facilitates peptide release [25–27]. In hypothalamic and nasal explant preparations, kisspeptin increased intracellular calcium levels and directly elicited GnRH release [26, 27]. However, few studies have demonstrated a direct effect of kisspeptin on gene transcription, mRNA stabilization, or peptide translation [48, 49]. In our studies, we showed that kisspeptin significantly increased AVP and OXY mRNA expression, yet it is possible that kisspeptin might also stimulate AVP and/or OXY peptide release from the PVN. This hypothesis was tested by measuring intracellular calcium currents following kisspeptin administration in two different neuronal cell lines derived from the PVN: IVB and H32. Our results showed that kisspeptin did not elicit calcium currents above baseline in these cell lines. These results were unexpected given the previously published reports of kisspeptin causing increased GnRH release from hypothalamic and nasal explants due to changes in calcium flux. One possibility is that the neuronal/glial environment surrounding the GnRH neurons, as in the case of an explant preparation, is critical for kisspeptin action. To our knowledge, there have been no previous studies examining kisspeptin action on intracellular calcium release in homogenous neuronal cell populations.

Overall, we conclude that it is unlikely kisspeptin directly regulates the HPA axis. However, given the positive effects on CRH, AVP, and OXY gene expressions in PVN-derived neuronal cells this possibility cannot be ruled out.

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