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Changes of plasma concentration and gene expression of *ghrelin* and *leptin* in rats receiving kisspeptin and morphine

Homayoun Khazali[1]*, Fariba Mahmoudi[2]

[1] Department of Animal Science and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran; [2] Department of Biology, Faculty of Sciences, University of Mohaghegh Ardabili, Ardabil, Iran.

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Abstract

Kisspeptin is a hypothalamic peptide which stimulates hypothalamus- pituitary- gonadal (HPG) axis. Morphine is an alkaloid which suppresses reproduction. Ghrelin and leptin are metabolic peptides which play role in relaying information to the HPG axis. In the present study, the interaction effects of kisspeptin and morphine were investigated on plasma and gene expression levels of *leptin* and *ghrelin*. Twenty adult male Wistar rats randomized in four groups were received injection of saline, kisspeptin (1.00 nmol), morphine (5.00 mg kg^{-1}) or Kisspeptin + morphine. Rats were received kisspeptin and morphine via third cerebral ventricular and subcutaneous injection, respectively. Ten male rats in two groups were received intravenous injection of saline or kisspeptin (7.50 nmol). Blood samples, hypothalamic and adipose tissue samples were collected. Plasma and gene expression levels of *ghrelin* and *leptin* were measured using the methods of enzyme-linked immunosorbent assay and real time-PCR, respectively. Morphine significantly increased plasma concentration and hypothalamic mRNA levels of *ghrelin* compared to saline while kisspeptin significantly decreased them compared to saline. Morphine significantly decreased plasma and mRNA levels of *leptin* in adipose tissue compared to saline, however, kisspeptin did not increase plasma and mRNA levels of *leptin* in adipose tissue compared to saline. Kisspeptin significantly decreased the effects of morphine on plasma concentration and hypothalamic gene expression levels of *ghrelin* compared to morphine alone, however, it did not affect morphine influence on plasma and *leptin* gene expression levels compared to morphine alone. Kisspeptin and morphine might partly be involved in the regulation of reproductive activity via regulation the metabolic hormones synthesis.

Introduction

Reproduction is a process which demands accurate regulation of metabolism. Infertility is caused often as a result of disturbances in negative and positive energy balance. Therefore, normal levels of metabolic hormones are crucial factors for controlling the release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH).[1,2] Opioids agonists and antagonists are potential factors for controlling pain and drug abuse clinically. In males and females, hypothalamus - pituitary- gonadal (HPG) axis is affected by opioids especially mu (μ) type receptor agonists including β-endorphin and morphine. Morphine is an extracted alkaloid from poppy plant which mimics endogenous β-endorphin actions and it induces hypogonadotropic hypogonadism (HH) via decreasing plasma testosterone levels predominantly due to suppressing GnRH/LH release.[3,4]

Kisspeptin is a neuropeptide which exerts its physiological action via binding to its receptor named GPR54 receptor. The third cerebral ventricular or intravenous (IV) injection of kisspeptin stimulates GnRH and LH secretion and testosterone.[5-7] Kisspeptin and GPR54 receptor expression levels alter under metabolic disorders.[8] In addition to its crucial role as a therapeutic target in reproduction, kisspeptin plays a key role in conveying metabolic information to hypothalamic GnRH neurons and metabolic status affects its expression level.[8]

Leptin is a 167 amino acid peptide which is synthesized in adipose tissue, hypothalamus and other organs. It plays

***Correspondence:**

Homayoun Khazali. PhD
Department of Animal Science and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
E-mail: h_khazali@sbu.ac.ir

a role in linking the nutritional status to reproductive axis.[9] It effects on GnRH and LH secretion is mediated via hypothalamic nuclei. Deficient in *leptin* gene results into infertility while injection of leptin stimulates GnRH/LH secretion via intra hypothalamic neuropeptides indirectly.[9,10] Also, it has been established that mutations of gene coding *leptin* results into decreasing *KiSS1* gene expression while injection of leptin stimulates kisspeptin synthesis.[10]

Ghrelin, a 28-amino acid orexigenic peptide, is an endogenous ligand for growth hormone secretagogue receptor (GHSR). It is produced in the hypothalamus, stomach, gonads and other peripheral organs.[11] It has been revealed that GnRH neurons express GHSR mRNA and they receive direct or indirect inputs from ghrelin neurons.[12] Ghrelin injection or food deprivation which causes an increase in plasma ghrelin levels contribute to decline in GnRH/LH and testosterone in both animals and humans.[13] Ghrelin exerts inhibitory effects on hypothalamic kisspeptin synthesis and injection of ghrelin decreases the stimulatory effects of kisspeptin signaling pathway on GnRH/LH release.[14,15]

The mu opioid receptors responsible for inhibiting GnRH neurons are not expressed in the ARC nucleus and the inhibitory influences of β-endorphin or morphine on the tonic release of GnRH/LH are exerted via indirect intra hypothalamic neurons.[16,17] Also, it has been established that kisspeptin has a crucial role in relaying the central or peripheral information to the reproductive axis.[18,19] In the present study, the interaction effects of kisspeptin and morphine were investigated on plasma and gene expression levels of hypothalamic *ghrelin* and *leptin* of adipose tissue in male rats.

Materials and Methods

Animals. In the present experimental study, 20 male Wistar rats weighing 230 - 250 g, provided by the Center of Neuroscience Research of Shahid Beheshti University (Tehran, Iran), were housed in the cages under controlled temperature (22.00 ± 2.00 ˚C) and light (12hr light/dark cycle, light on 07:00 hr). Animals had free access to food and water all the time. All procedures for the maintenance and the use of experimental animals were executed with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 80-23, revised 1996) and approved by the Ethical and Research Committee of Shahid Beheshti University, 1393: 13797.

Chemicals. Ketamine (Alfasan, Woerden, The Netherlands), xylazine (Alfasan), kisspeptin10 (Ana Spec Co., Fremont, USA), Morphine sulfate (Temad Co., Tehran, Iran), rat ghrelin and leptin kit (East Biopharm Co., Hangzhou, China), PureZol (Bio-Rad Laboratories, Hercules, USA), reverse transcriptase kit (Vivantis Co., Kuala Lumpur, Malaysia) and SYBR Green I (Takara Bio Inc., Shiga, Japan) were used in the present study.

Intracerebroventricular (ICV) cannulation and injections. The rats were anesthetized using intraperitoneal injection of 80.00 mg kg^{-1} ketamine and 10.00 mg kg^{-1} xylazine. Then a 22- gauge stainless cannula was implanted in the third cerebral ventricle according to the coordinates of the Paxinos and Watson Atlas (AP = – 2.30, ML = 0.00, DV = 6.50).[17]

Experimental groups. After one week recovery period, twenty rats in four groups received saline (3.00 µL, ICV) + saline (200 µL, subcutaneous (SC)), kisspeptin (1.00 nmol per 3.00 µL, ICV) + saline (200 µL, SC), saline (3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC) or kisspeptin (1.00 nmol per 3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC) respectively. The animals were received the acute single injection of drugs. For intra third cerebral ventricular injection, kisspeptin10 was dissolved in distilled water and was injected by a 27- gauge stainless steel injector which connected to Hamilton micro syringe by polyethylene-20 tubing at 09:00 - 9:30. For SC injection, morphine sulfate was dissolved in distilled water and was injected by an insulin syringe at 09:00 - 9:30. The second part of the study examined the effects of peripheral injections of kisspeptin on the *leptin* gene expression levels in the adipose tissue. Therefore, 10 rats in two group were received IV injections of saline or kisspeptin (7.50 nmol) at 09:00 - 9:30 via tail vein, respectively. During IV injection animals were housed in restrainer cage and they were not anesthetized during IV injection. The dose of drugs was chosen based on previous studies.[17,20]

Hormone assays. Blood samples were collected in a volume of 0.50 mL at 60 min following injections via tail vein. To prevent clotting, heparin was added to the samples. Then, blood samples were immediately centrifuged at 15 min at 3,000 rpm. Mean plasma ghrelin and leptin concentrations were measured using rat ghrelin and leptin kit and the method of enzyme-linked immunosorbent assay (ELISA; East Biopharm Co., Hangzhou, China).

Microdissections and real-time polymerase chain reaction (RT-PCR). Visceral adipose tissue and hypothalamic samples were dissected following deep anesthetizing of animals by injections of ketamine and xylazine. The rats were sacrificed and the brains were immediately removed. The brains were placed ventral side up, anterior coronal slices were cut from 1 mm anterior to optic chiasm. The slices were then dissected laterally up to the hypothalamic sulci and posterior coronal slices were cut posterior to the mammillary bodies.[17] Tissue samples were immediately frozen in liquid nitrogen and stored at – 80.00 ˚C. Total RNA was isolated from individual frozen samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method according to PureZol manufacturer instruction. Then, tissue samples were lysed with potent monophasic combination of phenol and the chaotropic agent guanidine isothiocyanate. Isolated RNA phase were then transported to a micro tube. The isolated

RNA were washed and purified using isopropanol and ethanol, respectively. The RNA sample was dissolved in diethyl pyrocarbonate (DEPC) and stored at – 80.00 ˚C. Changes in gene expression levels were determined using the real-time PCR detection system (Rotor Gene 6000; Corbette, Hilden, Germany) and SYBR Green I kit in a final volume of 25.00 µL according to manufacturer instruction. The *GAPDH* gene was used to normalize the values obtained for each sample. Reverse transcriptase step used temperatures were as follow: 65.00 ˚C for 5min, 42.00 ˚C for 60 min and 85.00 ˚C for 5 min according to manufacturer's instruction. The PCR cycling conditions were as follow: First denaturation 95.00 ˚C for 3 min, followed by 40 cycles of denaturation at 95.00 ˚C for 30 sec, annealing at 60.00 ˚C (*leptin*), 54.00 ˚C (*ghrelin*) and 58.00 ˚C (*GAPDH*) for 30 sec and extension at 72.00 ˚C for 30 sec, followed by final extension 72.00 ˚C for 7 min. Specific oligonucleotide sequences for forward and reverse primers are shown in Table 1. The leptin, ghrelin and GAPDH amplified products were 214, 132 and 112 base pairs, respectively. Calculation of relative expression levels of the target mRNAs were calculated by the equation $2^{-\Delta\Delta CT}$.

Table 1. Specific oligonucleotide sequences for forward and reverse primers.

Genes	Primers sequences
Leptin	F: 5′- GGATGACACCAAAACCCTCA -3′
	R: 5′- CATGAGCTATCTGCAGCACG -3′
Ghrelin	F: 5′- AATGCTCCCTTCGATGTTGG -3′
	R: 5′-CAGTGGTTACTTGTTAGCTGG -3′
GAPDH	F: 5′- AAGAAGGTGGTGAAGCAGGCATC -3′
	R: 5′-CGAAGGTGGAAGAGTGGGAGTTG-3′

Statistical analysis. The data were analyzed using SPSS Software (version 16.0; SPSS Inc., Chicago, USA) by unpaired *t*-test and one- way analysis of variance (ANOVA) followed by post hoc Tukey test. Significance was defined by $p \leq 0.05$. The results are presented as mean ± SEM.

Results

Effects of morphine and kisspeptin on mean plasma leptin and ghrelin concentration. Morphine + saline significantly increased mean plasma ghrelin concentration by 0.58 times compared to saline + saline group ($p \leq 0.05$, Fig. 1). Kisspeptin + saline or kisspeptin + morphine significantly decreased the mean plasma ghrelin concentration by 0.36 and 0.05 times compared to saline + saline group which this decrease only in Kisspeptin + saline group was statistically significant ($p \leq 0.05$, Fig. 1). Kisspeptin + morphine significantly decreased mean plasma ghrelin concentration by 0.40 times compared to saline + morphine group ($p \leq 0.05$, Fig.1). Kisspeptin + morphine significantly decreased mean plasma ghrelin concentration by 0.48 times compared to kisspeptin + saline group ($p \leq 0.05$, Fig. 1).

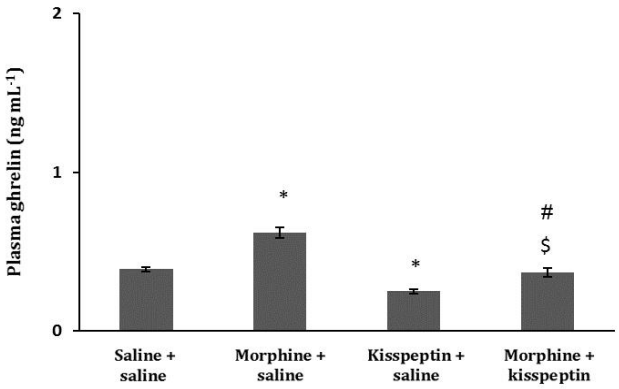

Fig. 1. The effects of saline (3.00 µL, ICV) + saline (200 µL, SC), saline (3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC), kisspeptin (1.00 nmol per 3.00 µL, ICV) + saline (200 µL, SC) or kisspeptin (1.00 nmol 3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC) on plasma ghrelin hormone concentration. ICV: Intra-cerebroventricular and SC: Subcutaneous. Data are presented as mean ± SEM and significance was defined by $p \leq 0.05$. * Compared to Saline + saline; $ Compared to morphine + saline; and # Compared to kisspeptin + saline.

Mean plasma leptin concentration was significantly decreased in morphine + saline or kisspeptin + morphine groups by 0.18 or 0.16 compared to saline + saline group, respectively ($p \leq 0.05$, Fig. 2). Kisspeptin + saline increased mean plasma leptin concentration by 0.10, but this decrease was not statistically significant in comparison with saline + saline group (Fig. 2). Mean plasma leptin concentration was significantly decreased in kisspeptin + morphine groups by 0.23 compared to kisspeptin + saline group ($p \leq 0.05$, Fig. 2). A significant increase in mean plasma leptin concentration was not observed between morphine + saline and kisspeptin + morphine groups (Fig. 2).

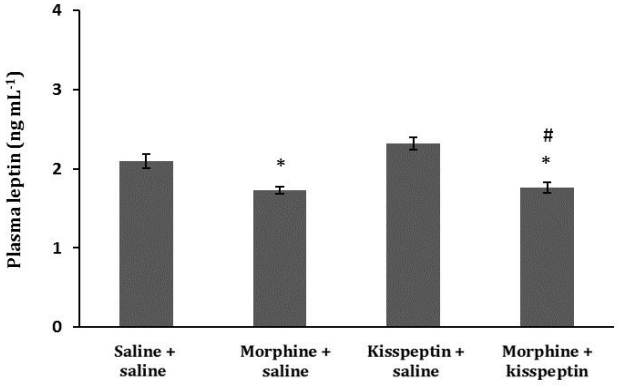

Fig. 2. The effects of saline (3.00 µL, ICV) + saline (200 µL, SC), saline (3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC), kisspeptin (1.00 nmol per 3.00 µL, ICV) + saline (200 µL, SC) or kisspeptin (1.00 nmol per 3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC) on plasma leptin hormone concentration. ICV: Intra-cerebroventricular and SC: Subcutaneous. Data are presented as mean ± SEM and significance was defined by $p \leq 0.05$. * Compared to Saline + saline; and # Compared to kisspeptin + saline.

Effects of morphine and kisspeptin on *ghrelin* gene expression in the hypothalamus. Mean relative *ghrelin* gene expression was significantly increased by 1.35 times in morphine + saline group compared to saline + saline group ($p \leq 0.05$, Fig. 3). Kisspeptin + saline significantly decreased mean relative *ghrelin* gene expression by 0.43 compared to saline + saline group ($p \leq 0.05$, Fig. 3). Injections of kisspeptin + morphine increased the mean relative *ghrelin* gene expression by 0.21 times compared to saline + saline group (Fig. 3), but this increase was not statistically significant. Injections of kisspeptin + morphine significantly decreased the mean relative *ghrelin* gene expression by 0.48 times compared to morphine + saline group ($p \leq 0.05$, Fig. 3). Injections of kisspeptin + morphine significantly increased the mean relative *ghrelin* gene expression by 1.12 times compared to kisspeptin + saline group ($p \leq 0.05$, Fig. 3).

Effects of morphine and kisspeptin on *leptin* gene expression in the adipose tissue. Mean relative *leptin* gene expression was significantly decreased by 0.32 times in morphine + saline group compared to saline + saline group ($p \leq 0.05$, Fig. 3). Kisspeptin + saline increased mean relative *leptin* gene expression by 0.07 compared to saline + saline group, but this increase was not statistically significant (Fig. 3). Injections of kisspeptin + morphine decreased the mean relative *leptin* gene expression by 0.24 times compared to saline + saline group, but this decrease was not statistically significant (Fig. 3). Injections of kisspeptin + morphine increased the mean relative *leptin* gene expression by 0.12 times compared to morphine + saline group which this increase was not statistically significant (Fig. 3).

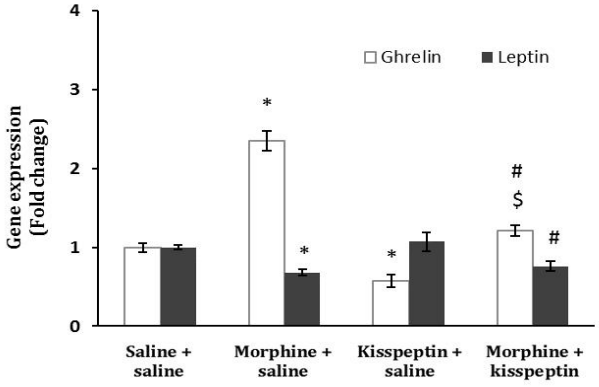

Fig. 3. The effects of saline (3.00 µL, ICV) + saline (200 µL, SC), saline (3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC), kisspeptin (1.00 nmol per 3.00 µL, ICV) + saline (200 µL, SC) or kisspeptin (1.00 nmol per 3.00 µL, ICV) + morphine (5.00 mg kg^{-1}, 200 µL, SC) on *ghrelin* gene expression in the hypothalamus and *leptin* gene expression in the adipose tissue. ICV: Intra-cerebroventricular and SC: Subcutaneous. Data are presented as mean ± SEM and significance was defined by $p \leq 0.05$. * Compared to Saline + saline; $ Compared to morphine + saline; and # Compared to kisspeptin + saline.

Injections of kisspeptin + morphine significantly decreased the mean relative *leptin* gene expression by 0.29 times compared to kisspeptin + saline group ($p \leq 0.05$, Fig. 3).

Effects of IV injections of kisspeptin on hypothalamic *ghrelin* and adipose tissue gene expression of *leptin*. Intravenous injection of kisspeptin significantly decreased the mean relative hypothalamic *ghrelin* gene expression by 0.70 times compared to saline group ($p \leq 0.05$, Fig. 4). The IV injection of kisspeptin increased the mean relative *leptin* gene expression in the adipose tissue by 0.21 compared to saline group but this increase was not statistically significant (Fig. 4).

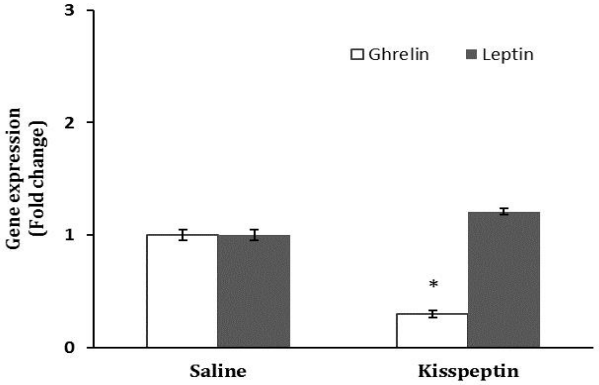

Fig. 4. The effects of intravenous IV injections of kisspeptin (7.50 nmol) on hypothalamic *ghrelin* and adipose tissue gene expression of *leptin*. Data are presented as mean ± SEM and significance was defined by $p \leq 0.05$. * Compared to saline.

Discussion

The results of the present study showed that subcutaneous administration of morphine significantly increased *ghrelin* mRNA in the hypothalamus and it significantly decreased *leptin* mRNA in the adipose tissue of male rats. The used doses of morphine and kisspeptin in the present study were chosen based on the results of our previous studies which established that third cerebral ventricular injection of 1nmol kisspeptin significantly increased mean plasma LH and testosterone concentration compared to control Wistar rats while the subcutaneous injection of 5.00 mg kg^{-1} morphine significantly decreased the mean plasma LH and testosterone concentration compared to control Wistar rats.[17,20] For the first time, the effects of morphine were investigated on *ghrelin* and *leptin* gene expression. However, the present results were consistent with the previous studies which investigated the hormonal interaction of opioids, ghrelin and leptin signaling systems in the regulation of GnRH/LH release.[21-24]

Lanfranco *et al.* demonstrated that IV injection of 1.00 and 2.00 µg kg^{-1} ghrelin inhibited the stimulatory effects of IV injection of 0.10 mg kg^{-1} naloxone (opioid receptor

antagonist) on LH secretion in men.[21] Ogata *et al.* established that intracerebroventricular injection of ghrelin and IV injection of naloxone increased the mean plasma LH concentration and LH pulse frequency in comparison with ghrelin injection in rats.[22] In fact their results suggested that ghrelin neurons might be involved in mediating inhibitory influences of opioids especially mu receptor agonists on HPG axis in rats.[22] Investigators showed that beta endorphin has a lipolytic activity in isolated human fat cells.[23] In weight loss methods for treatment of female obesity a reverse relationship was observed between the plasma levels of leptin and beta endorphin.[24]

Previous studies demonstrated the inhibitory effects of ghrelin and stimulatory effects of leptin on kisspeptin neural pathways and GnRH/LH hormones release. The results of the present study for the first time showed that IV or third cerebral ventricular administration of kisspeptin did not significantly increase *leptin* mRNA levels in the adipose tissue of male rats, however, it significantly decreased ghrelin mRNA. The present results were in consistent with the researches which demonstrated that central injection of kisspeptin declined serum ghrelin concentrations in male rats.[25] Also, injection of peptide234 (GPR54 receptor antagonist) abolished the inhibitory influences of kisspeptin on serum ghrelin secretion.[25] To interpret the present results one could propose an increase in plasma growth hormone (GH) levels following kisspeptin injections that may play a role in suppressing ghrelin synthesis. Both ghrelin and kisspeptin exert stimulatory effects on GH secretion.[11,26] High concentration of plasma GH in turn inhibits ghrelin secretion via a negative feedback mechanism.[27] Therefore, kisspeptin may decline ghrelin mRNA partly via the stimulation of GH secretion.

Previous studies demonstrated that peripheral injection of kisspeptin did not affect mean plasma leptin levels in fed or fasted monkeys.[15] In rats, kisspeptin hinders the process of lipid accumulation by declining lipogenesis and promoting lipolysis. Also, its injection increased leptin secretion from adipocytes.[28] Previous studies established that defects of gene expressing leptin or resistance to leptin induced by consuming fat regimen resulted in down regulation of *Kiss1* gene expression in the ARC nucleus of hypothalamus in mice and Wistar rats.[29,30] Hence, they suggested that leptin induced upstream of kisspeptin neurons to control GnRH/LH release.[29,30] Castellano *et al.* demonstrated that hypogonadism and lower *Kiss1* gene expression of diabetic rats were improved by central injections of leptin.[31] Also, in the present study, the interaction between kisspeptin and morphine were determined on *leptin* and *ghrelin* gene expression in the adipose tissue and hypothalamus. Kisspeptin blocked the stimulatory effects of morphine on *ghrelin* gene expression but it did not affect morphine influences on *leptin* mRNA levels. To better understand the action of kisspeptin/GPR54 signaling systems upstream ghrelin neurons further studies are needed using higher doses of kisspeptin rather than its effective dose for stimulating HPG axis. Also, for investigating the exact role of leptin and ghrelin in mediating the opioid or kisspeptin effects on HPG, further studies are needed using ghrelin and leptin receptor antagonists.

In conclusion, morphine may be involved in the regulation of reproduction partly via controlling the synthesis of ghrelin in hypothalamus and leptin in adipose tissue. However, kisspeptin signaling pathway is more likely to be involved in the regulation of reproductive process via controlling hypothalamic ghrelin neural activity rather than leptin. Suppressive effects of kisspeptin on ghrelin might be mediated partly via decreasing hypo-thalamic opioid neural activity and the present results suggested that kisspeptin and opioid signaling pathways might interact at hypothalamic levels to control ghrelin mRNA levels.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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