Original Article

Effects of abnormal levels of testosterone on the expression of hypothalamic kisspeptin in male rats

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Abstract:

Introduction:
Testosterone regulates the secretion of gonadotropin-releasing hormone (GnRH) and gonadotropin through a negative feedback mechanism in males. The effects of testosterone are mediated by upstream steroid-sensitive neurons because GnRH neurons do not express androgen receptors. Kisspeptin neurons have been considered the missing link in the feedback control of GnRH neurons. Moreover, the central effects of supra-physiological levels of testosterone are not well understood. The present study was therefore conducted to determine the effect of abnormal levels of testosterone on the hypothalamic expression of kisspeptin in male rats.

Material and Methods:
Three weeks following a single injection of 100, 250 and 500 mg/kg body weight of testosterone undecanoate, the blood testosterone concentration was measured to ensure that the experimental treatments have been effective in creating the desired supra-physiological levels of testosterone. The relative expression of kisspeptin was evaluated in the anterior and posterior hypothalamus using qRT-PCR and compared with the kisspeptin expression in the controlled and gonadectomized (GDX) rats (n=6 in each group).

Results:
Gonadectomy was found to significantly increase the kisspeptin expression in the posterior hypothalamus in the rats; however, no significant differences were observed in the kisspeptin expression in posterior hypothalamus between the control group and the GDX rats treated with different concentrations of testosterone. On the other hand, supra-physiological levels of testosterone increased the kisspeptin expression in the anterior hypothalamus compared to in the controls.

Conclusion:
Different physiological concentrations caused by abnormal testosterone levels appear to differently affect hypothalamic kisspeptin neurons.

Keywords: Testosterone, Kisspeptin, Hypothalamus, Rat

Introduction

Reproduction is the essential process for preserving species, which is controlled by a complicated network of regulatory signals. The hypothalamus-pituitary-gonad or gonadotrophic axis is the origin and/or the main target of regulatory agents in mammals. Hypothalamic GnRH-expressing neurons stimulate the release of

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gonadotrophins from the pituitary gland, and these hormones in turn cause the synthesis of gonadal sex steroids. Sex hormones regulate the activity of GnRH neurons by exerting positive and negative feedback effects. In males, testosterone regulates the secretion of GnRH and thereby gonadotropin through a negative feedback mechanism (1). GnRH neurons do not express androgen receptors (2). The effects of this sex steroid must therefore be mediated by steroid-sensitive neurons upstream from GnRH neurons.

Two independent studies conducted in 2003 reported loss-of-function mutations in the kisspeptin receptor (Kiss1r) gene in patients with hypogonadotropic hypogonadism (3-4). These findings highlighted, for the first time, the fundamental role of kisspeptin signaling in controlling the basic aspects of reproductive functions. Since the discovery of the key role of kisspeptin in human fertility more than ten years ago, many studies have shown that kisspeptin controls fertility and the onset of puberty in all mammals by activating the Kiss1r in GnRH neurons. Initial findings suggest that kisspeptin is a powerful stimulus for luteinizing hormone (LH) secretion in some species (5). It is not therefore unexpected that kisspeptin is the strongest ever-known stimulant of GnRH neurons (6-7). Most of GnRH neurons (approximately 85%) were found to express the Kiss1r in adult rodents using different methods (6 and 8-9).

Discovering kisspeptin as the strongest ever-known stimulant of GnRH neurons caused a significant progress in human knowledge about the neurohormonal regulation of reproduction. Given that most of kisspeptin neurons express steroid hormone receptors, they are considered the missing link in the feedback control of GnRH neurons by sex steroids (10). In rodent hypothalamus, kisspeptin-expressing neurons are mainly in the arcuate nuclei and the anteroventral periventricular nuclei (AVPV) nuclei (11). Arcuate kisspeptin neurons are believed to play a role in the negative feedback mechanism of sex steroids and kisspeptin neurons in the AVPV nuclei in the positive feedback mechanisms (10). Although the knowledge about the mechanisms involved in the steroidal positive feedback effect is swiftly expanding, the mechanism contributing to the negative feedback effect of sex hormones is yet to be understood (12).

Numerous pathological circumstances contribute to the supra-physiological secretion of testosterone. Moreover, using androgen constitutes a major health problem in athletes (13). Abnormal levels of androgen have been reported to negatively affect many of the body organs, including the cardiovascular system and the central nervous system (14). The hypothalamic effects of testosterone on the brain are probably the best known impact of this gonadal steroid. Given that supra-physiological levels of testosterone may act differently from its physiological concentrations, the kispeptin neural network was assumed in the present study to be affected by hormonal interventions and function as the main regulator of GnRH neurons. The present research was therefore carried out to determine the effects of abnormal concentrations of testosterone on the kisspeptin expression in the posterior and anterior hypothalamus in male rats.

Materials and Methods

The present basic experimental study was conducted on 30 adult male Sprague-Dawley rats (Rattus Norvegicus), weighing 220-270 g and as old as 3-4 months. The study rats were randomly selected from the proliferation center of laboratory animals in Shahid Beheshti University of Medical Sciences, Tehran, Iran. These rats were kept in the 12:12 light-dark cycle at 22 ˚C, with light starting from 7:30 am. All stages of the experiment were performed between 10 am and 1 pm according to the ethical principles of laboratory animal research in the Faculty of Biological Sciences and Technology of Shahid Beheshti University (Ethics Code: D/920/1010).

Testosterone undecanoate (Nebido, Bayer Pharma, Berlin, Germany) was used to create fixed testosterone concentrations during the trial. A single intramuscular injection of testosterone undecanoate to rats had already been shown to be able to stabilize the blood testosterone level for at least four weeks (15). To determine the effect of supra-physiological concentrations of testosterone, 30 rats were randomly assigned to five experimental groups (n=6 in each group) and their hypothalamic kisspeptin expression was investigated. The experimental groups comprised the control group (CTL), a GDX group, a GDX group receiving 100 mg per kg body weight of testosterone undecanoate (GDX+T100), a GDX group receiving 250 mg per kg body weight of testosterone undecanoate (GTX+T250) and a GDX group receiving 500 mg per kg body weight of testosterone undecanoate (GDX+T500).

The rats were anesthetized in all the five groups using a combination of ketamine (Alfasan, 100 mg/kg) and xylazine (Alfasan, 10 mg/kg). A vertical 1-cm-long incision was then created at the end of the abdomen and the testicles were drawn out of the scrotum. Testicular arteries were sutured, the testicles were separated and the abdominal incision was sutured in all groups except the controls. The suture site was then sprayed with oxytetracycline to prevent potential infections. Three days following the surgery and after ensuring the health and relative recovery of the rats, testosterone undecanoate was injected into their thigh muscle at the cited concentrations. The GDX group was injected with sesame oil. Three weeks following the trials, blood samples were collected from all the rats and they were then slaughtered.

The serum testosterone concentration of all the study rats was measured using the Testosterone (125I) RIA KIT (#RK-61M IZOTOP, Hungary).

After the trial, all the rats were anesthetized using CO_2, beheaded using guillotines and their brain was immediately extracted from their skull. The hypothalamus tissue was separated after creating the first coronal incision on optic chiasma and the second coronal incision behind the mammillary bodies. To remove hypothalamus, two sagittal incisions were made 3 mm apart from each side of the third ventricle and a horizontal incision below the hypothalamus border. To separate the anterior hypothalamus from the posterior hypothalamus, the third coronal incision was made in the middle of the optic tract (16). After separating the posterior and anterior hypothalamus, they were placed in liquid nitrogen for 24 hours and were then kept at -80 ˚C in the freezer.

The YTzol Pure RNA solution (#YT9062, Yekta Tajhiz Azma, Iran) was used for extracting RNA from the tissue samples. NanoDrop was also used to measure the concentration of all the extracted RNA samples. Moreover, 1 µL of a few RNA samples were placed on the 1% agarose gel in the horizontal electrophoresis to ensure the sample quality. To eliminate any potential genomic contamination, the extracted RNA samples in all groups were treated with DNase I (#EN051, Thermo, USA), which was immediately followed by synthesizing the first cDNA strand for each sample using Easy cDNA Synthesis Kits (#A101162, Pars Toos, Iran). Furthermore, the instruction recommended for using the kits were followed in all the stages

Given the data available in the database (NCBI) and the sequence of the encoding gene of Kiss1, specific primers were designed for the rats using Allele ID 7. Beta-actin was used as an internal controller and its specific primer was also designed. The sequence of the primers designed was as follows:

Kiss1 (sence)
5′- TGATCTCGCTGGCTTCTTGGC-3′
Kiss1 (antisense)
5′- GGGTTCAGGGTTCACCACAGG-3′
Beta-actin (sense)
5′- TCTATCCTGGCCTCACTGTC-3′
Beta-actin (antisense)
5′- AACGCAGCTCAGTAACAGTCC-3′

The specific primers and the first strand of cDNA were utilized as the pattern to investigate the kisspeptin expression in the experimental groups using qRT-PCR. The enzyme RealQ Plus 2x Master Mix Green (#A325402, Ampliqon, Denmark) was also used for the investigation cited. All of the reactions involved closed 20 µL containers in the ABI StepOne Real-Time PCR system (Applied Biosystems, USA). The thermal cycle used included 10 minutes at 95 ˚C, 40 cycles at 95 ˚C for 30 seconds, 56 ˚C for 30 seconds and 72 ˚C for 30 seconds.

At the end of the cycle-time of the equipment, the melting curves drawn for the samples were scrutinized to ensure a single peak for each sample. In addition, the qRT-PCR products were randomly placed on the 1% agarose gel in the horizontal electrophoresis system to ensure that the band width created is acceptable. The qRT-PCR data were ultimately calculated using the Ct comparison method and comparing target gene expressions with the internal control gene expressions using the 2-ΔΔCT formula (17).

Given that the present study used six rats in each group, the data associated with the relative kisspeptin expression in the experimental groups compared with that in the controls were analyzed using the non-parametric Kruskal–Wallis test. GraphPad Prism 7.03 was also used to analyze the data and draw the diagrams. The data were reported as mean±standard deviation and $P<0.05$ was set as the level of statistical significance.

Results

The present research examined the effect of supra-physiological concentrations of testosterone on the hypothalamic expression of kisspeptin. Three weeks following the testosterone treatment, the blood testosterone concentration was measured to ensure that testosterone undecanoate has created the desired supra-physiological concentrations in the experimental groups. As observed in Table 1, gonadectomy significantly reduced serum testosterone concentrations, and dose-dependent testosterone treatment increased the concentration of this steroid hormone. Given that kisspeptin neurons are expressed only in the arcuate nuclei and the AVPV nuclei of rodent hypothalamus, the hypothalamus tissue was cut in the present study in such a way that the arcuate nuclei lie in the posterior hypothalamus and the AVPV nuclei in the anterior hypothalamus. The posterior hypothalamus tissue was therefore considered the arcuate nucleus and the anterior hypothalamus tissue the AVPV nucleus (16). The results obtained using the qRT-PCR method showed that gonadectomy significantly increases (by over 2600%) the Kiss1 mRNA expression in the posterior hypothalamus of the rats; nevertheless, no significant differences were observed between the control group and the gonadectomized rats treated with different testosterone concentrations in terms of the expression of kisspeptin in the posterior hypothalamus (Table 2).

The present findings also suggested that supra-physiological testosterone concentrations significantly increase the Kiss mRNA expression in the anterior hypothalamus compared with the control group (Table 3). Although gonadectomy reduced the kisspepting expression in the anterior hypothalamus by approximately 50%, the reduction was statistically insignificant.

Table 1: The mean blood concentration of testosterone in the experimental groups

	Experimental Group				
	CTL	GDX	GDX+T100	GDX+T250	GDX+T500
Mean Testosterone Concentration (nmol/L	0.73±5.26	0.03±0.59	2.93±15.30	2.17±30.73	8.49±45.60

Table 2: The relative kisspeptin expression in the posterior hypothalamus of the rats compared with the controls

	Experimental Group			
	GDX	GDX+T100	GDX+T250	GDX+T500
Relative kisspeptin expression in the posterior hypothalamus (mean±SD)_	2671±625*	185±49ns	181±6ns	145±28ns

*: Data were reported as mean±SD. P<0.05, ns: not significant

Table 3: The relative kisspeptin expression in the anterior hypothalamus of the rats compared with the controls

	Experimental Group			
	GDX	GDX+T100	GDX+T250	GDX+T500
Relative kisspepting expression in anterior hypothalamus (mean±SD)_	51±1ns	764±79*	829±43*	643±31*

*: Data were reported as mean±SD. P<0.05, ns: not significant

Discussion

The present research findings revealed that supra-physiological levels of testosterone significantly increase (by about 700%) the kisspeptin expression in the anterior portion of hypothalamus in the study rats compared with in the control group. On the other hand, treating the GDX rats with abnormal testosterone concentrations inhibits the increase in the kisspeptin expression in the posterior hypothalamus. Overwhelming evidence suggests that sex steroids differently regulate the kisspeptin expression in two different neuronal populations. Gonadectomy increases the kisspeptin expression in the arcuate nuclei and post-gonadectomy testosterone treatments inhibit the Kiss1 mRNA expression in the arcuate nuclei of rats (18). Similar results were also reported in male rats (9). It is worth noting that gonadectomy reduces the Kiss1 mRNA expression in males' AVPV, while testosterone increases their expression (18).

Arcuate nucleus kisspeptin neurons are believed to contribute to the negative feedback mechanism associated with steroids and the GnRH pulse generation. Sex steroids clearly inhibit arcuate nucleus kisspeptin neurons in many species. In line with this effect, the increased post-gonadectomy kisspeptin expression is associated with the increased LH secretion, potentially as an attempt for generating more sex steroids. Furthermore, the central administration of the kisspeptin antagonist inhibits the LH secretion in rats and GDX rats as well as the LH pulse secretion in female monkeys and ovariectomized (OVX) ewes (19). These findings reaffirm the association between stimulating the post-gonadectomy GnRH/LH secretion and kisspeptin signaling.

The path or paths by which arcuate nucleus kisspeptin neurons regulate GnRH neurons are not clear. Although the terminals of arcuate nucleus kisspeptin neurons extend towards POA (20), there is little evidence suggesting their direct relationship with the body of GnRH neurons. Moreover, kisspeptin neurons do not appear to be directly linked with the terminal of GnRH neurons in the outer region of the median eminence, since 1) very few kisspeptin neurons have been found in this area (21); 2) there is no microscopic evidence suggesting direct relationships between presynaptic kisspeptin inputs and GnRH terminals (22) and 3) kisspeptin neurons in mice and rats did not absorb the peripherally injected fluorogold (20 and 22). The anatomic relationship between arcuate nucleus kisspeptin neurons and GnRH neurons therefore remains unknown. Moreover, kisspeptin neurons in the AVPV nuclei appear to play a key role in activating GnRH neurons for the preovulatory surge

of GnRH/LH in female rodents (23), since female AVPV nuclei contain 10-20 times as many kisspeptin neurons as males do (24-25), and they are shown using immunohistochemical methods and investigations of cFos that they are activated simultaneously with the surge (26-27), and directly activate GnRH neurons (28). The physiological role of this type of neurons is unclear, as males do not naturally produce positive steroidal feedbacks and they may therefore contribute to other testosterone-related processes; nevertheless, the present study results clearly showed that high testosterone concentrations can cause a pseudo-surge kisspeptin expression in the AVPV nuclei in male rats, which naturally occurs in females upon the preovulatory GnRH surge.

Although most of kisspeptin neurons in the arcuate nuclei and AVPV nuclei express estrogen receptors, including ERα and ERβ in males (18 and 29), the mediator mechanisms of testosterone effects appear to be different in the two populations of kisspeptin cells. In The AVPV, testosterone effects appear to be mediated by ERα or ERβ, since the steroidal treatment can completely imitate the testosterone effect; however, in dihydrotestosterone, which cannot be aromatized to estradiol, these effects were negligible, indicating the absence of the androgen receptor role. In the rats knock-outed rats for ERα, the testosterone effect on regulating the AVPV nucleus kisspeptin was maintained, disproving the mediating role of ERα and suggesting the possible contribution of ERβ. In males' arcuate nuclei, both dihydrotestosterone and estradiol are able of imitating inhibitory effects of testosterone (18). The regulation of the arcuate nucleus kisspeptin can therefore be mediated by both estrogen receptors and androgen receptors. The inhibitory effect of testosterone on arcuate nucleus kisspeptin is not therefore disrupted in male rats lacking ERα or androgen receptors, since the absence of one of the two can be compensated by the presence of the other.

Conclusion

According to the previous findings and the present results, elevated testosterone levels in the brain can suddenly increase the AVPV nuclei kisspeptin expression following the aromatization to estradiol. Moreover, supra-physiological testosterone concentrations inhibit the increase in the post-gonadectomy kisspeptin expression through the arcuate nucleus androgen receptors.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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